INVESTIGATIONS IN THE FIELD OF FLUOROMETRIC AND PHOTOMETRIC TITRATIONS AND DETERMINATIONS

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SUMMARY

The investigations reported in this thesis involve: the construction of a new instrument for fluorometric titrations, the photometric titration of vanadium, and two procedures for the automated photometric determination of iron in blood serum. At first glance the areas of investigation may seem somewhat unrelated, but each resulted from initial work with photometric titrations. A need was seen to develop the potentially more sensitive fluorometric titration procedures. Such procedures would offer an entirely different array of fluorogenic reagents with which to improve not only sensitivity but selectivity. It was found that the instruments available were not fully satisfactory, and, therefore, design and construction of an instrument for the purpose of monitoring fluorometric titrations was initiated.

The development of the automated techniques for iron came as the result of investigations of a new reagent for iron as a possible photometric titrant. While the reagent was not useful for this purpose it had significant advantages over existing iron reagents and it was decided to pursue investigations into its use in the important area of serum iron assay.

In order to meet the needs to effectively perform titrations with fluorogenic agents an instrument was devised that allows such titrations at high sensitivity and without interference from ambient light. The instrument employs a modified double beam system in which the reference signal is used to compensate for changes in exciter lamp output and

fluctuations in the photomultiplier. A chopper system blocks light to both the reference and sample beam 163 times each second and triggers an electronic compensation for photomultiplier dark current and any ambient light which might reach the photomultiplier.

Evaluation of the instrument showed it to meet a number of criteria established prior to its initial design and necessary for a satisfactory titrator whether photometric or fluorometric.

A procedure was developed for the determination of vanadium (v) by photometric titration with PAR. The absorbance is monitored at 542 nm and the endpoint determined either graphically or by least squares approximations applied to two linear line segments. This procedure allows the assay of vanadium in final solution concentrations of less than 1 μg/ml with an accuracy and precision of approximately 1%. The procedure uses a minimum number of reagents and steps and can be used with either a standard spectrophotometer or a phototitrator.

Molybdenum is one of the elements most frequently accompanying vanadium and not easily separated from it. Therefore, investigations were carried out to determine vanadium in the presence of excesses of molybdenum. It was found that by applying substiochiometric masking with EDTA, up to an approximately 18,800 fold excess of molybdenum could be tolerated.

The reagent ferrozine has been applied to two procedures which will give high volume analyses of iron and total iron binding capacity. One of the instruments was the continuous flow processor, AutoAnalyzer, produced by Technicon Instrument Company and the other was a discrete analyzer (DSA) manufactured by Beckman Instrument Company.

Both techniques offer linear response up to 300 μg/100m iron with coefficients of variation of approximately 2 to 3%. The AutoAnalyzer procedure offers the advantages of giving 100% steady state at the rate of 40 samples per hour with good sensitivity and the capability of approximately doubling sensitivity by using double dialysis, changing the ratio of sample to diluent lines, and decreasing the rate of analysis.

The procedure allows analyses at the rate of 120 tests per hour with a precision and accuracy approximately the same as that of the AutoAnalyzer. Copper does not interfere at normal concentrations, but a masking technique is described for copper which is effective up to 500 μg/100 ml and possibly higher. In addition, a different approach has been taken to the preparation of samples for the determination of total iron binding capacity with a resulting improvement in speed and sample conservation.

PART ONE

A NEW INSTRUMENT FOR FLUORESCENT TITRATIONS

CHAPTER I

INTRODUCTION

A. General Remarks

Luminescence is the name given to the phenomenon of light emission by a substance. Several types of luminescence can be distinguished according to the mechanism of the emission. Chemiluminescence is caused by the release in the form of light of a portion of the energy liberated during a chemical reaction. In triboluminescence a portion of the crystal lattice energy is given off as light when crystals of certain materials, e.g. cube sugar, are disrupted by grinding or breaking. Incandescence is the phenomenon where light is emitted when a substance is heated. The phenomenon of immediate interest here is the emission of light following the absorption of radiant energy by a system. When the emission takes place in a very short time (about 10^{-8} sec. or less) after the occurrence of absorption the process is called fluorescence. If emission is delayed, the name phosphorescence is employed.

When light energy is absorbed an electron is moved from a lower energy state, usually the ground state, to an excited state. In the case of fluorescence this absorption step is immediately followed by the return of the electron to a lower level with the simultaneous release of a quantum of light energy (photon) corresponding to the particular electron transition. The energy of the exciting radiation must be at least equal to that of the emitted radiation and usually is higher in prac-

tical applications. This picture of electron orbital change is highly simplified because energy exchanges with rotational and/or vibrational levels are superimposed, which results in the fact that the absorption and emission spectra do not consist of lines, but of rather broad bands.

Fluorescence was observed in minerals and other natural products long before G. G. Stokes gave an explanation of the process in terms of an interrelationship between light absorbed and light emitted. Stokes is also to be credited with coining the name "fluorescence" which was derived from the mineral fluorspar, which shows the phenomenon to a rather striking degree, emitting an intense blue white light upon irradiation with ultraviolet light.

Almost 100 years passed after the phenomenon was basically explained until fluorescence spectroscopy started to become an established field of investigation in 1945 (1). The growth of this field since then, however, has been remarkable and today hundreds of analytical methods are based on fluorescence. There are two principal reasons for the popularity of fluorescent methods, (a) an inherent high level of sensitivity, and (b) a high degree of selectivity. Small amounts of some substances can emit relatively high intensity light which is measured against practically no light at all. This situation can be contrasted to absorption photometry where small intensity changes must be measured against a high intensity background. Selectivity is favored because the number of compounds which fluoresce is limited, and discrimination between these compounds can frequently be made by appropriate choice of the wavelength of exciting light and careful isolation of the emitted radiation.

These and several other advantages of fluorometry as an analytical

tool are limited by two facts of extreme practical importance. One is that the relation between emitted intensity and concentration of the emitting species is linear only at very low concentrations (see Appendix). Thus one has to operate with "bent" calibration curves. As such this does not make a determination impossible but it is not a preferred situation. The other limiting condition arises from the phenomenon of quenching. Fluorescent output or emission is often decreased, that is, quenched by compounds which are otherwise optically inactive. Unless such quenchers are completely matched in effect and concentration in the standards, wrong results are to be expected. The method of standard additions can cope with the quenching problem but is no help in case of curvature of the calibration plot. Such a situation when dealing with absolute measurements is frequently found and often an improvement can be achieved by switching to a method where only changes in a parameter are of importance, rather than absolute values. The advantages of such an approach in the form of a linear titration are well known for photometric determinations versus photometric titrations, turbidimetric determinations versus turbidimetric titrations, conductimetric determinations versus conductimetric titrations, and others.

The situation in case of fluorescence is analogous. If a linear fluorometric titration is compared with a fluorometric determination it can be seen immediately that a less than strict proportionality between fluorescence intensity and concentration of emitting species is of far lower consequence and great curvature can be tolerated as long as the slope change of the titration curve at the end point is sufficiently pronounced to permit clear location of that point. Extrapolation of

straight line portions as in the case of photometric titrations is less important here because one is almost always working within concentration ranges where considerations of equilibrium shifts are of minor importance. Quenching, unless existing to a degree which lowers the level of emitted intensity below practical ranges, is also of minor influence. The overall fluorescent level is lower and will result in a change in slope of the titration curve but will have no influence on the location of the end point break. Considering these advantages it is rather surprising that linear fluorometric titrations have not found greater analytical applications.

Fluorometric titrations have been applied in many areas, including acid base, redox, precipitation and complexation titrations. However, most of these titrations are logarithmic ones and the end point is detected visually. A special setup is generally recommended in order to facilitate the observation of changes in fluorescent color or intensity. A typical device is described by Rudolph et al. (2). An outline of the recent range of applications is shown in the Handbook of Analytical Chemistry published in 1963 (3) which cites references for 47 titrations for metal ions alone in which a change in fluorescence is used to indicate the end point. This list is merely an indication of the developments which have been made in a short time and active research in this field has produced many more such titrations since then.

B. Instrumental Titration vs. Visual Titration

Just as the application of a photometric titrator adds a new dimension to visual titrations in which a change in color is observed,

the application of a fluorotitrator increases the analytical potential of fluorescent titrations. In the following sections some of the types of titrations and situations are discussed where the use of a fluoro-titrator has great advantage over visual endpoint determination and in some cases can make an otherwise infeasible titration possible.

1. Self Indicating Titrations

In this class of titrations no indicator, in the classical sense of the term, is used. Instead the increase or decrease of fluorescent intensity is monitored as the titration proceeds. Such titrations can be used for the assay of very low concentrations of certain constituents, by making use of the chromogenic agents otherwise used as indicators. In this case the "indicator" is used as the titrant and fluorescence will begin either to appear or to disappear as soon as titrant is added, and the process will continue until all of the element or compound being titrated has reacted. Fluorescent readings are plotted against the volume of titrant added and straight lines are drawn through two sets of points. The intersection of these two lines is taken as the endpoint. In the case of unfavorable equilibrium conditions a pronounced curvature will occur in the region around the end point. Under such circumstances the initial straight portion and the straight portion far beyond the end point are taken and extrapolated towards intersection. If curvature in the titration curve is due to the fact that fluorescence intensity is not strictly proportional to the concentration of the species involved such extrapolation is not permitted. It is then necessary to have a pronounced break in the curve at the endpoint.

Where in the case of complexometric titrations, the stability

constant of the complex between the chromogenic titrant and species to be titrated is too small to allow a good titration the possibility exists to add the chromogenic agent in excess and then to titrate with another complex former. Thus a self indicating system has been created and the excess of chromogenic agent shifts the equilibrium of the weak complex to the favorable side. This technique can be carried one step further and the species to be titrated can be complexed or reacted with an excess of a chromogenic agent which forms a very stable complex but for one reason or another is not an acceptable titrant, e.g., the kinetics of the reaction are unfavorable. This new species can then be titrated with a suitable titrant also using the self indicating technique.

2. Minor Constituents and Sequential Titrations

In certain cases where impurities or minor constituents react with the "titrant" in a manner similar to that of the major component, these constituents can be pretitrated and the titration continued to give an accurate determination of the major constituent. In somewhat the same manner it is possible to titrate two components to successive endpoints. In either case it would not be possible to detect the initial change in fluorescence if the titration were monitored visually.

3. Limitations Imposed by the Visual Range

The visual range of color perception is limited to a range of approximately 400-700 nm with the sensitivity falling off dramatically near each of these limits. With the wider ranges of photodetectors an instrument is not bounded by such limits. Titrations can, therefore, be carried out when the radiant energy emitted by the solution is above

or below the range which will give a visual response.

Even when the emitted radiation is within the visual range, large differences exist in the abilities of individuals to see changes in fluorescent colors or intensities. Under such circumstances an instrumental technique which utilizes a photodetector will provide more precise and reliable means of estimating the endpoint.

4. Background Color or Fluorescence

If the solution to be titrated has a background color or fluorescent contaminant, it is difficult, at best, to detect the appearance or disappearance of the indicator fluorescence. For example, in the titration of the fluorescing calcium-calcein complex, if fluorecein (a common impurity in calcein) is present, a residual fluorescence will remain which makes locating the endpoint (normally signalled by a sharp change from fluorescence to non-fluorescence) difficult to see. Other sources of optical interferences include certain plastics which fluoresce strongly, residues from detergents, and reflections of the exciting light off the titration vessel.

5. Unfavorable Equilibrium Conditions

Titrations in which the endpoint is determined visually are generally limited to "step titrations" where a true indicator system is used. The accuracy and precision of the endpoint depend upon the affinity of the indicator for the titrand, the rate of response of the indicator and the concentration levels at which the titration is carried out. As an example a complexometric titration may be discussed where a metal (M) is titrated with a ligand (L), and an indicator (I) is used which fluoresces in the free form. Upon addition of the indi-

ator the following complexation reaction will take place:

$$M + I^* = MI \qquad (1)$$

Any fluorescing species will here and later be marked by an asterisk. The stability constant of the complex formed is:

$$K_1 = \frac{[MI]}{[M]\,[I^*]} \qquad (2)$$

Upon addition of titrant the free metal will react according to:

$$M + L \rightleftarrows ML \qquad (3)$$

This complex has the stability constant:

$$K_2 = \frac{[ML]}{[M]\,[L]} \qquad (4)$$

In order for the titration to proceed smoothly K_2 has to be larger than K_1. Under these conditions the free metal ion will be complexed until it is all consumed, then the indicator bound metal will be attacked and the reaction:

$$MI + L \rightleftarrows MI + I^* \qquad (5)$$

will occur. The equilibrium constant for this reaction is:

$$K_3 = \frac{[ML]\,[I^*]}{[MI]\,[L]} = \frac{K_2}{K_1} \qquad (6)$$

Two conditions must be met in order that the endpoint is sharp and located correctly: (1) the stability of the indicator metal complex must

be sufficiently high so that practically no free indicator is left, otherwise, some fluorescence will prevail during the course of the titration, and (2) the constant K_2 must be sufficiently larger than K_1 so that at the endpoint the metal is taken away without the use of an excess of ligand. When either or both of the requirements are not fulfilled, the sharpness of the endpoint will be decreased. Often visual location of the endpoint is made difficult or even impossible. If followed instrumentally, however, the requirements are much less stringent. Similar discussions can be made for other types of titrations such as acid-base, redox, and precipitation.

C. Photometric Titration vs. Photometric Determination

As has already been noted in the cases of quenching and non-linearity of response at higher concentrations, a fluorometric titration has significant advantages over a fluorometric determination. Some other advantages of consequence are included in the following sections.

1. Instrument Stability

A certain degree of random instability and noise are of less consequence in a titration than in a technique where a single reading is taken. In a titration a number of readings are necessary to define the titration curve, or in the case of self-indicating titrations, to construct the two line segments required to define the endpoint. These multiple readings will average out much of the fluctuation and make the final result less likely to be in error.

2. Standard Curves

Standard curves which must be prepared by measuring the fluores-

cence of a series of standards and then plotting the measurements against concentration may not only be "bent" but if incorrectly applied can result in significant errors. If there are even minor changes in conditions between the standards and the test solutions, the resulting fluorescence outputs of two like concentrations can be quite different and result in an under or over estimation of the unknown concentration. On the other hand, in a titrimetric procedure the titrant is either a standard or has been standardized eliminating the need for a standard curve. Thus in a determination a sometimes large number of parameters must be repeatedly established in exactly the same fashion or to the same degree. In a titration the parameters must be only established to the extent that they are within the limits where the reaction proceeds correctly and the only requirement is that they remain constant during the course of a titration. Such conditions are much more easily achieved. The vanadium determination dealt with in Part Two is a very instructive example for such a situation.

D. Existing Instruments

A review of the literature discloses a number of instruments which have been designed to monitor fluorescent titration using photodetection devices. These instruments vary widely in design and in their approach to design problems and requirements inherent in fluorescent assay. The simplest of the instruments was designed by Vecerek and Skovronsky (4) and includes a light source, a 100 ml titration vessel, a filter and photocell. All of the components are arranged in a straight line with the filter serving to select only the emitted radiation from the sample.

A galvanometer is used to measure the output of the photocell.

In another design developed by Clements and Sergeant (5) the photodetector is placed at right angle to the incident radiation and both primary and secondary filters are used. A 125-watt mercury lamp provides the exciting energy and a cadmium-selenide photocell converts the sample emission into an electrical signal which is amplified with a pair of high gain transistors and displayed on a 100 micro-ampere meter. The stated sensitivity is that "one-drop endpoints" are normally possible using 0.01 M titrants. This is ambiguous since it gives no information on what sort of titrants and indicators were used. This system is limited to macro-titrations.

Stolyarov, et al. (6) also used amplification of the output signal. In this instrument the emission from the sample passes through two secondary filters and strikes a phototube. Its output is amplified by a vacuum tube and displayed using a mirror galvanometer. In contrast to the previous instrument this one was designed for microtitrations.

Howerton and Wasitewski (7) designed an instrument called the "Titracolormat" which makes use of the sensitivity of a photomultiplier tube for detection of radiant energy. The instrument is the most versatile of all reviewed, but depends upon a tungsten source which limits the excitation range. The photomultiplier is connected directly to a microammeter and no use is made of amplification.

Murakami and Kimura (8), and Borle and Briggs (9) modified existing fluorometers to accept a buret or other means of introducing titrant and a stirrer.

Two commercial instruments are currently being marketed one by

Fiske Associates (10) the other Corning Instruments (11). Both instruments are designed to exclusively monitor the fluorescent titration of calcium using calcein as the indicator. This single purpose design makes the instruments essentially useless for any of the many other fluorescence titrations which are available.

Each of the instruments reviewed has deficiencies which limit its usefulness to monitor fluorescent titrations. The lack of stable energy sources and limited sensitivity and the necessity of placing the sample in a shielded compartment to prevent interference from ambient light are the most frequent limitations.

E Statement of the Problem

The problem was to design and construct an instrument which could be used to take advantage of the potential analytical benefits found in fluorescent titrations. Such an instrument required more than a simple modification of already available instruments to meet certain requirements which would make it an effective tool for monitoring various types of fluorescent titrations. These requirements include:

1. Stability

Once a titration is initiated, it is difficult and undesirable (and in many cases impossible) to interrupt the operation repeatedly to check the initial set-point. An instrument should be sufficiently free from drift so that the entire titration can be completed without a significant change in the set-point. Also, noise should be minimized so that the readout device, either a meter or recorder, can be read to the limit of its sensitivity. This means that a technique must be devised

to stabilize the output of the rather high intensity sources required or to compensate for their fluctuation. This is especially important for those sources which involve a plasma discharge. These lamps are inherently less stable than, for example, a low intensity incandescent source, which can be operated by a very stable voltage supply such as a battery.

2. Stray Light

Stray light in this discussion will be restricted to unwanted radiant energy produced by the exciting source. This potential interference can be reduced or eliminated by the use of a primary filter to limit the wavelength range of the light striking the sample and a secondary filter to isolate the wavelength range which strikes the photodetector to that produced by the sample.

Further steps which aid in the elimination of stray light are to make measurements of sample emission at a 90^{o} angle to the incident radiation, and to shield against unfavorable internal reflections within the instrument.

3. Ambient Light

One of the primary considerations in the proposal of a new instrument was that it be insensitive to ambient light without enclosing the titration vessel. This would allow the analyst to follow the titration visually and use various pieces of apparatus without introducing random or systematic errors due to room light.

4. Versatility and Convenience

The instrument must be designed in such a way that various wavelengths can be selected for different titrations, that either micro or

macro titrations can be performed with the appropriate burets and titration vessels, and that ancillary devices such as temperature control can be added. The instrument must be easy to use so that any technician can operate it without a great deal of instruction and training.

5. Sensitivity

The amount of light energy derived from a fluorescent sample is relatively small. The instrument must be sufficiently sensitive to measure this emission in titrations where the fluorescence increases or decreases. It should allow measurements in the concentration ranges where the output fluorescence is proportional to the concentration of the fluorochrome (see Appendix). While, as mentioned earlier, this is not essential for successful titration, it would enhance the versatility of the instrument by allowing it to be used for conventional fluorometric measurements.

F. Preliminary Investigations

A proposal was made for a new instrument which would satisfy all of the requirements. The initial design was based upon a double-beam system in which a rotating chopper was to be used to separate the initial beam from a high intensity exciting source into sample and reference beams. The reference beam and the emitted energy from the sample would be recombined at a photomultiplier. The resulting signals would be amplified and the sample signal measured as a percentage of the reference signal, the latter being defined as 100%. Since the ambient light was considered as a constant background and since the electronics were designed to measure only light pulses, it was felt that freedom from the

influence of ambient light could be achieved.

After construction of the instrument was started, it became obvious that some of the initial approaches were either unsuitable or added unnecessary complexity to the system without improving performance. These revelations led to a number of modifications of the original design. Among these modifications were changes in electronics and optics to compensate for the fact that the ambient background is, in most cases, not constant but is pulsed at an even multiple of 60 Hz. Others included changes in the design of the optical reference beam to improve simplicity and in the phasing system to reduce cost.

CHAPTER II

THE FLUOROTITRATOR

The construction of the instrument was completed in two stages: First, the electronics package was constructed and then secondly the optics portion. The completed components are shown in the photograph in Figure 1. Throughout design and construction, the guiding principles were simplicity, low cost, and high performance. If compromises had to be made, they were made in favor of performance.

A. Optical and Mechanical Assembly

The optical portion of the instrument is shown diagramatically in Figure 2 and consists of an 85-watt mercury lamp (A), a focusing lens (B), a primary filter (C), a rotating chopper (D) which is detailed in Figure 4, a lens system (E and E'), a titration vessel (F), a collimating tube (G) with a secondary filter (H) mounted midway between the collimating lenses, an RCA 931A photomultiplier (I), a variable occluder (J) which is detailed in Figure 5, a fiber optic bundle (K), a 2.5 v prefocused lamp (L), and two photosensitive diodes (M and M'). Figure 3 is a photograph of the optical assembly.

A modified double-beam system is maintained in which the rotating chopper directs radiant energy either into the sample or reference pathways. The sample pathway begins at the chopper. The radiant energy from the source passes through an open sector of the chopper, through lenses E and E' and is focused on the center of the titration vessel. The energy

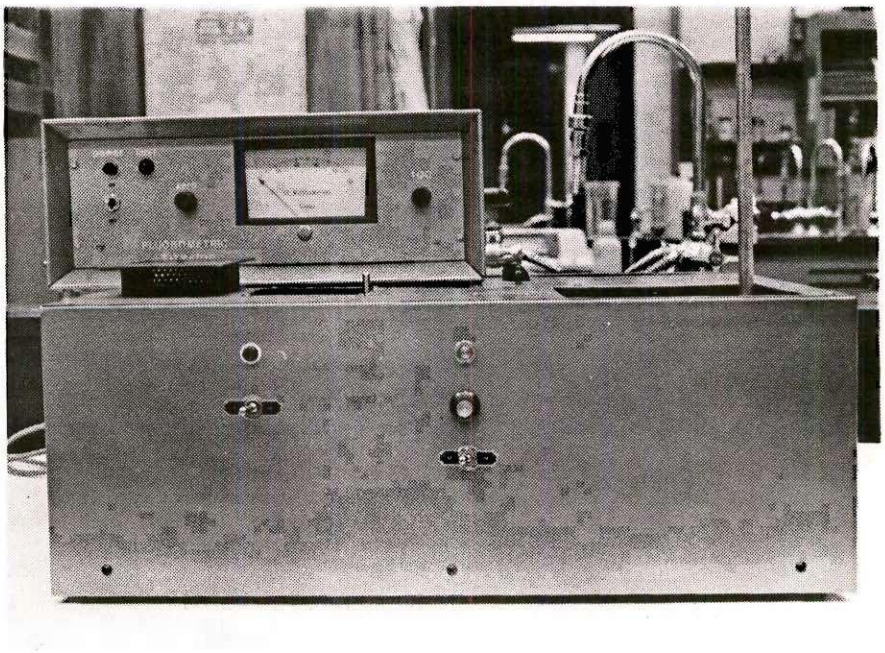
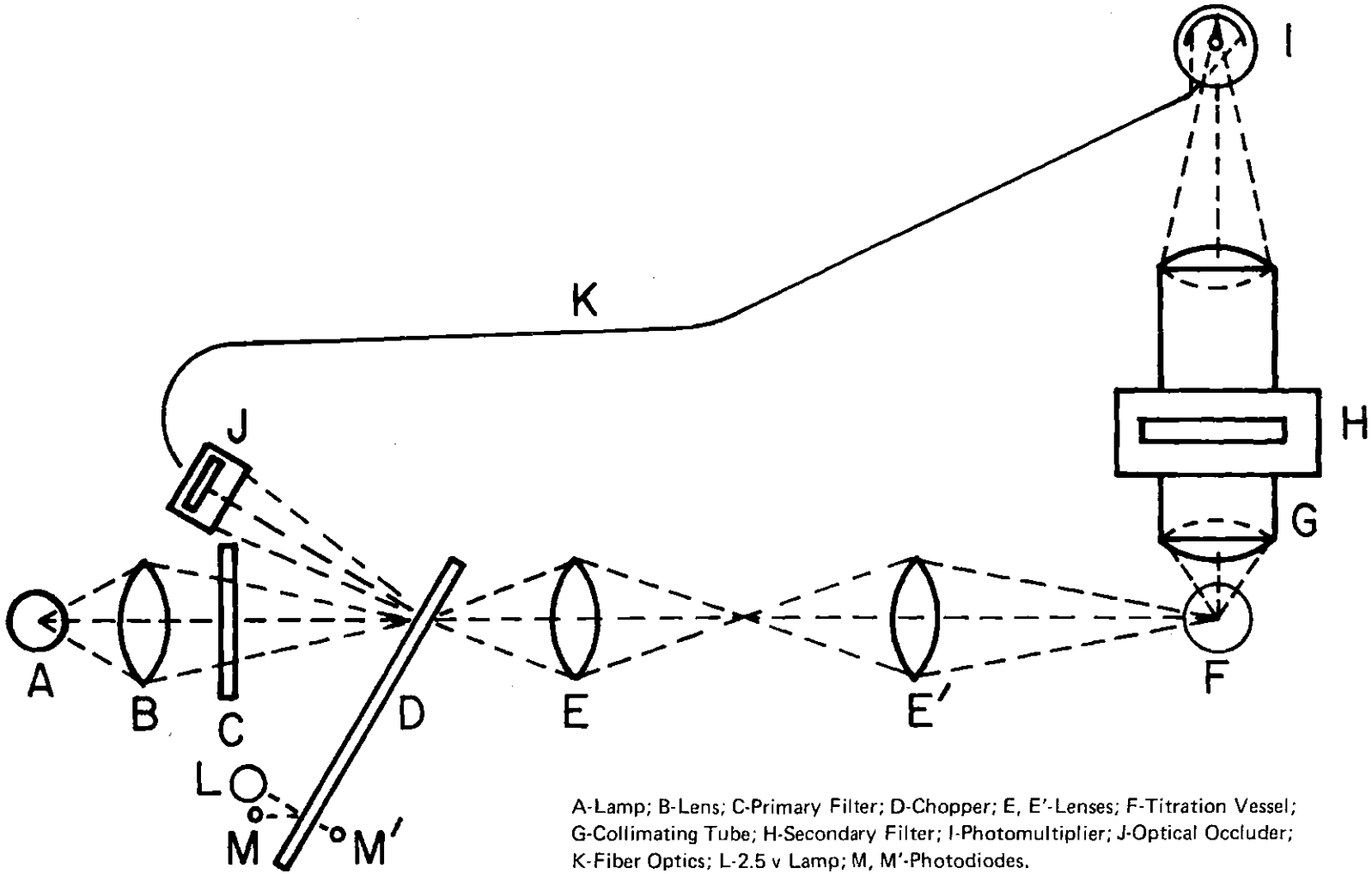
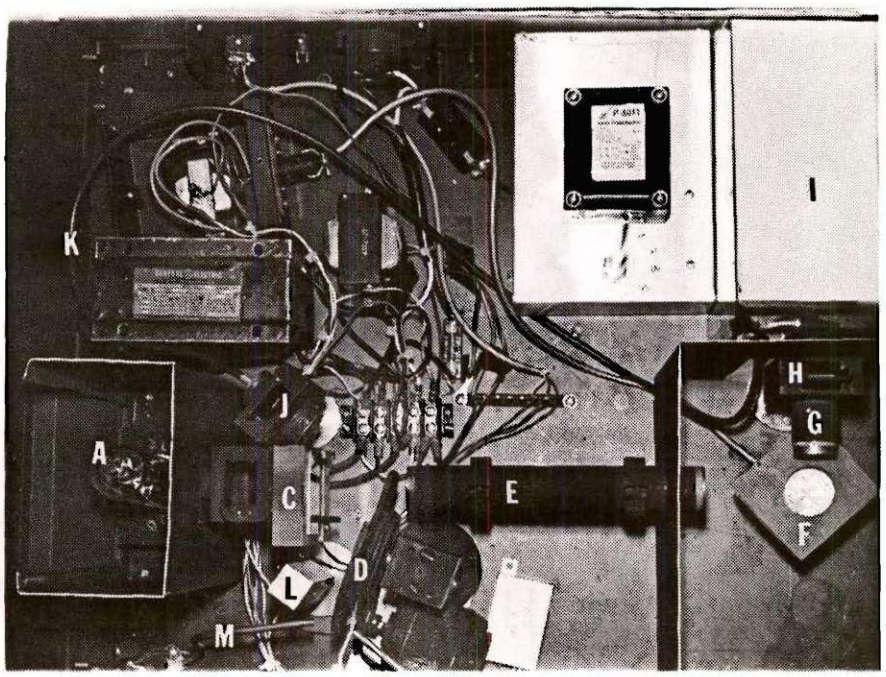


Figure 1. Photograph of the Completed Fluorotitrator.



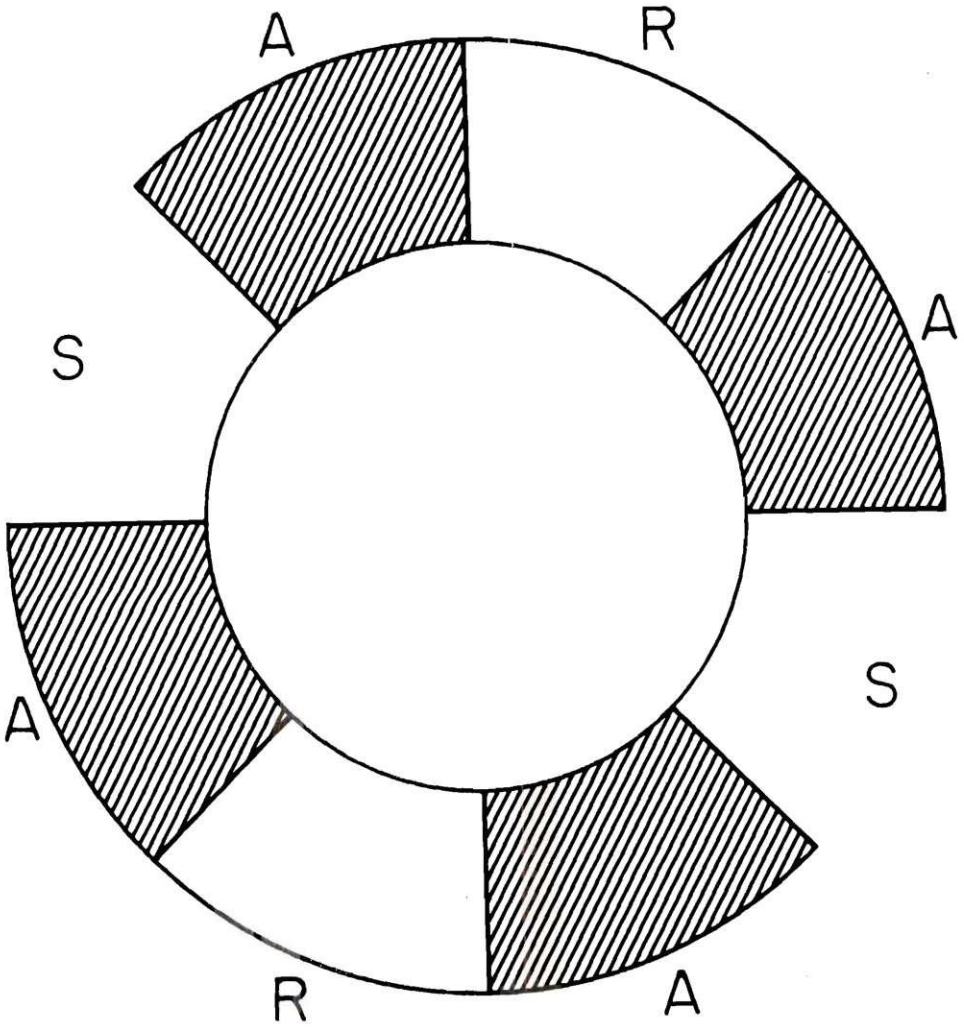
A-Lamp; B-Lens; C-Primary Filter; D-Chopper; E, E'-Lenses; F-Titration Vessel; G-Collimating Tube; H-Secondary Filter; I-Photomultiplier; J-Optical Occluder; K-Fiber Optics; L-2.5 v Lamp; M, M'-Photodiodes.

Figure 2. Optical Diagram of Fluorotitrator.



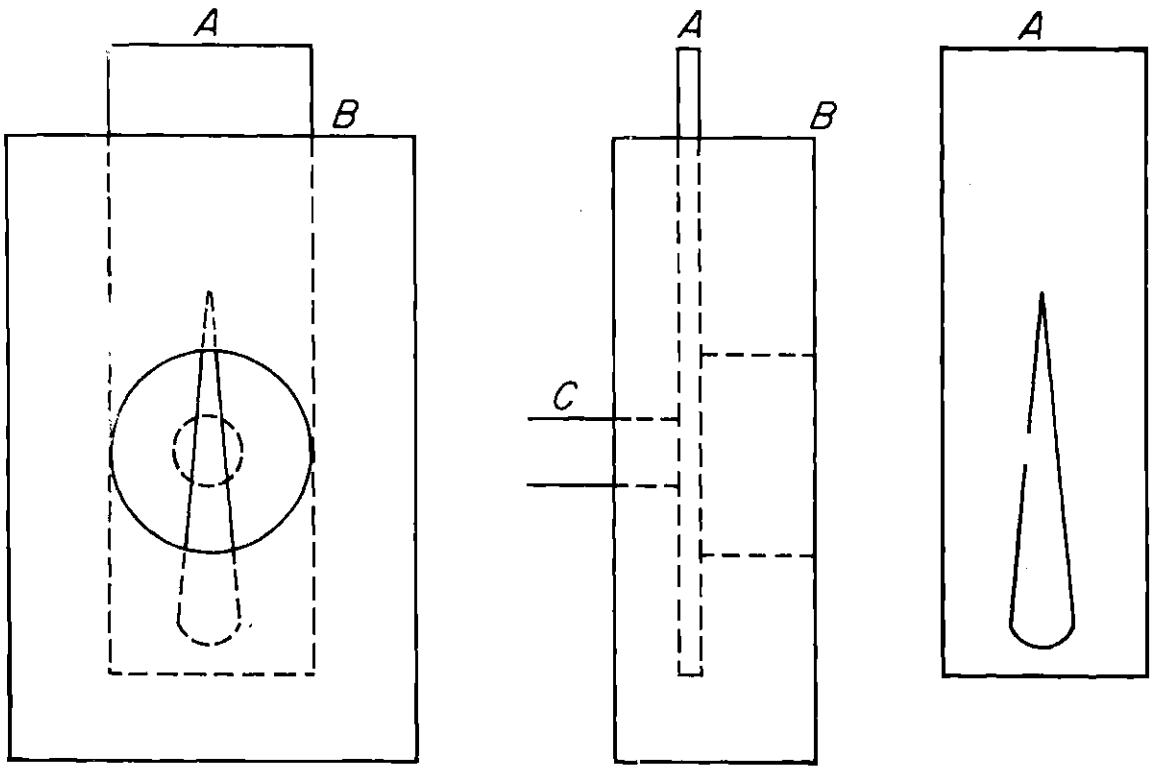
A—Lamp; C—Primary Filter; D—Chopper; E—Lens System; F—Magnetic Stirrer and holder for Titration Vessel; G—Collimating Tube; H—Secondary filter; I—Photomultiplier;Compartment; J—Optical Occluder; K—Fiber Optics; L—2.5v Lamp; M—Photodiode Holders.

Figure 3. Photograph of the Optical Portion of the Fluorotitrator.



Sector S– is open and allows energy from the mercury lamp to reach the sample.
Sector A– is nonreflecting so that no energy reaches the detector from the source.
Sector R– is a reflecting surface which causes energy to pass through the reference path.

Figure 4. Rotating Chopper.



A—Moveable plate containing a wedge shaped appeture.
B—Mounting block
C—Fiber optic bundle.

The moveable appeture allows continuously variable illumination of from 0 to 100% of the surface of the fiber optics.

Figure 5. Detail of the Optical Occluder.

emitted by the sample passes through the collimator and the secondary filter and is finally focused on the cathode of a photomultiplier. Radiant energy reflected from the polished sectors of the chopper is picked up by the fiber optic bundle. The variable occluder allows the intensity of light which reaches the photomultiplier via the fiber optics to be adjusted to a level comparable to that produced by the sample.

Since sample and reference beams strike the same spot on the photomultiplier surface alternately, it is necessary to use some technique to maintain the proper phase relationship and to trigger the electronic operations. An optical system was selected which consists of a low intensity light source and two photodiodes with a very short response time. This system is placed parallel to the exciting radiation path so that every action taken on the exciting radiation is simultaneously taken on the phasing light. This arrangement guarantees that the system will stay in phase no matter what changes occur in the chopper speed. The only requirement is that the chopper maintain its physical symmetry.

B. Electronic Assembly

The electronic portion of the instrument is composed of a preamplifier, three integrating amplifiers, and three switches. The switches are tied into the optical phasing system which insures that when a given sector of the chopper is in the exciting light beam the proper integrating amplifier is activated and performs its function. The functions of these integrators are: to (a) provide electronic compensation for ambient light which strikes the photomultiplier, (b) to

operate an automatic gain control on the preamplifier to compensate for changes in source intensity and photomultiplier output, and (c) to amplify the average output caused by the sample so that it can be read on a meter or recorder.

The function diagram of the electronics is shown in Figure 6. The most important features of this design are the four operational amplifiers (A1 - A4). (Burr Brown 3112/12C) A1 is the preamplifier which serves as a current to voltage converter and receives all of the output of the photomultiplier tube. A2 is the central component of a negative feedback loop for compensation for ambient light levels. A3 amplifies the reference signal and provides a voltage output which compensates for source or photomultiplier fluctuation. A4, the sample amplifier, amplifies the signal from the sample and provides a signal output for either a 100 microammeter or a 1 millivolt recorder, both of which can be operated simultaneously.

The operation of these systems can be best described by a discussion of the sequence of events which occur at each position of the chopper, (Figure 4). When the open sector of the chopper is in the incident beam, the exciting light strikes the sample and the subsequent emission if picked up by the photomultiplier, the output of the photomultiplier is monitored by the current to voltage converter A1. Simultaneously, light from the phasing lamp passes through the second sector and causes the photodiode PD2 to conduct, and thereby to operate the switch driver Q7-8 which closes the signal switch Q4. The 2 μF capacitor C4 associated with the signal amplifier A4 then begins to charge up to the voltage output of amplifier A1. At the next chopper position a dark sector blocks

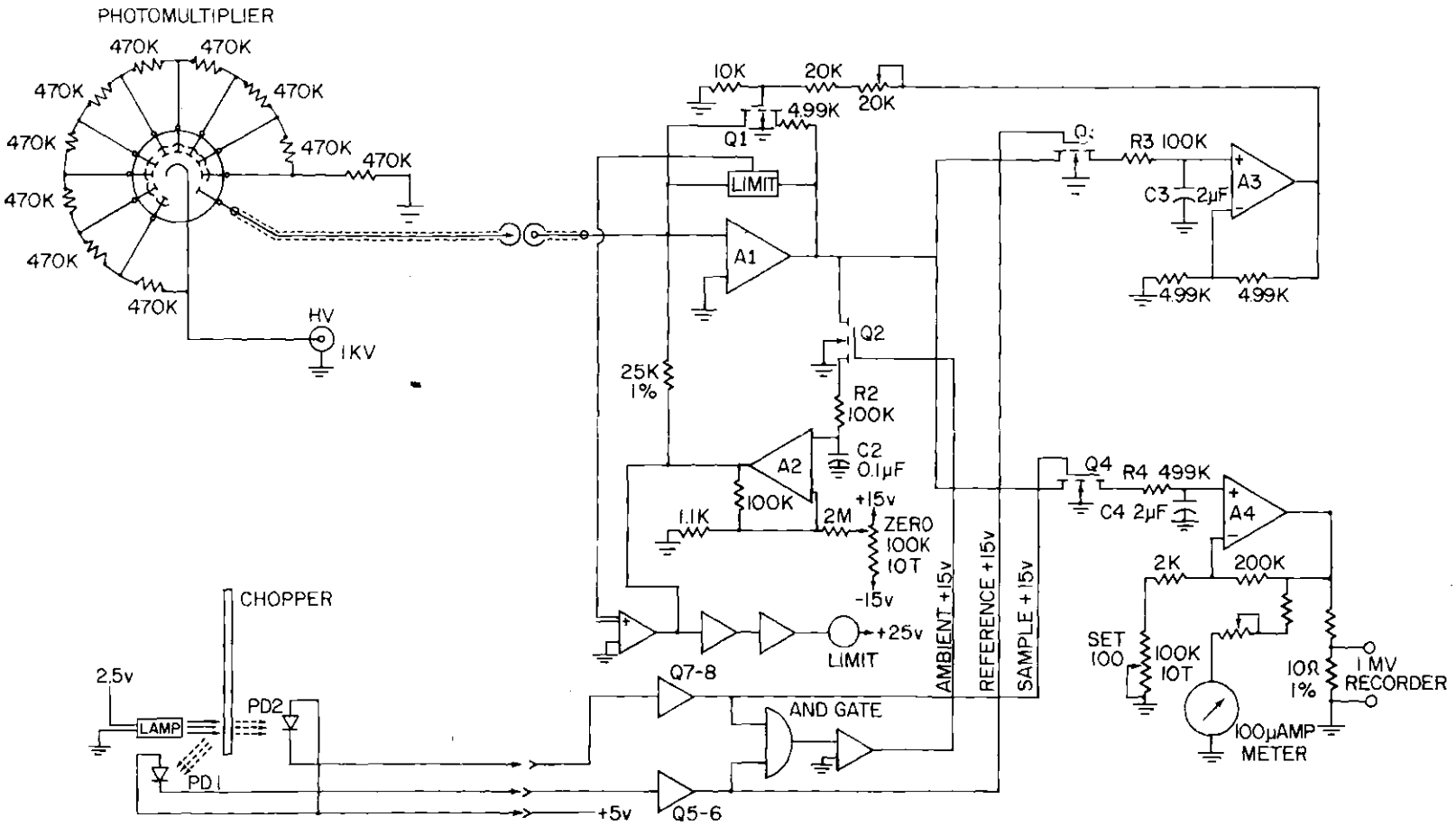


Figure 6. Electronic Diagram of the Fluorotitrator.

off all of the light. During this period the photomultiplier detects only the fraction of ambient light which passes through the secondary filter. Neither of the photodiodes are illuminated so both the sample and reference switch driver outputs are at -15v. The like signals are allowed to pass through an "and" gate and operate the ambient switch driver Q9-11 which closes switch Q2 and allows the 0.1 μF capacitor C2 of the ambient amplifier A2 to begin charging to the output of A1.

Finally, when a reflecting surface of the chopper is in the incident beam, light is reflected through the occluder and fiber optic path to the photomultiplier. Simultaneously, light from the phasing lamp is reflected to photodiode PD1 allowing it to conduct and drive the reference switch driver Q5-6. The 2 μF capacitor C3 associated with reference amplifier A3 begins to charge up to the output of A1. As the chopper rotates this sequence is repeated until each of the capacitors is charged to the output of the amplifier A1 for its respective phase of operation; this makes each of the operational amplifiers an integrator, with an integration period equal to the RC constant for the associated capacitor. The integration period of the ambient amplifier A2 is the shortest, approximately 0.01 seconds. Its averaged signal is fed back to the summing point of the preamplifier A1 which forces the sum of the currents at this point to equal zero. This has the effect of cancelling photomultiplier dark current and average ambient light effect.

The reference integrator A3 has a time constant of 0.2 seconds. This system feeds back a constant voltage signal through resistor R1 and the field effect transitor Q1 which acts as a voltage controlled resistor. The output of A1 is given by the relation $E_o = I_{pm} R_f$ where R_f = 4.99K +

R_{Q1} (E_o = voltage out of A1, I_{pm} = photomultiplier current, R_f = feedback resistance). If the reference output is decreased then R_{Q1} and E_o are increased. Should the lamp intensity or the photomultiplier output vary, the gain on preamplifier A1 will be increased or decreased to maintain a constant reference output and this will assure that the sample output will be independent of lamp and photomultiplier fluctuations.

A protection or limiting circuit is also included which plays no role in normal operations, but if the current from the photomultiplier exceeds the normal operating levels the limiting network will conduct and protect A1.

Finally, the capacitor associated with sample amplifier A4 is charged to the output of A1 (this requires about 1 sec.). The voltage is then amplified and measured on a 0-100 micro amp-meter or a 1 millivolt recorder. The gain of A4 is adjusted with the "SET 100" control and can be varied for amplification factors from 2 to 50.

C. Discussion of Design Parameters

The initial proposal for the fluorotitrator included a number of requirements. These requirements were considered necessary for an instrument to be useful as a general laboratory tool. In most instances a combination of optical and electronic approaches were taken to fulfill the requirements.

1. Stability

The stability of the instrument over extended periods of operation is enhanced by the fact that the light source and photomultiplier are constantly monitored as a unit, and any changes in output are automatical-

ly corrected by electronic adjustment of the preamplifier gain.

Stability of the instrument readout in terms of lack of random meter movement is achieved by the integration step build into the signal-handling system. The chopper rotates at 2440 rpm's which means that during the one second integration period the output sees 81.$\dot{3}$ individual signals from the sample.

The combination of these two stabilizing features allows the use of a pulsed light source (the mercury lamp fires at 120 Hz) which would have been impossible with the design mentioned in the preliminary experiments.

2. Stray Light

Stray radiation from the exciter lamp which might reach the photodetector is minimized or eliminated by the conventional arrangement of the photodetector at a 90^{o} angle from the incident radiation and by the proper choice of primary and secondary filters.

3. Ambient Light

The possibility of operation with the sample exposed to room light is one of the most significant features of the instrument. External light can only reach the photomultiplier by passing through the collimator tube (G) and the interference filter. The instrument case prevents room light with its intense visible component from entering the collimator directly and thus saturation of the photomultiplier is impossible. The inside of the collimator tube is painted with optical black to aid exclusion of indirect radiation. The use of a narrow band-pass secondary filter further limits that fraction of ambient light which reaches the photomultiplier. Finally, the chopper and electronic system samples and

corrects for the remaining fraction of ambient light four times in each revolution of the chopper, 162.6̇ times each second.

4. Convenience and Simplicity

The operational convenience of the instrument can be seen in a brief description of the steps necessary to perform a titration.

a. Insert primary and secondary filters required for the titration to be performed.

b. Turn on the power switch and allow 5 to 10 minutes for the mercury lamp to reach maximum intensity.

c. Turn on the electronics power switch.

d. With a solution which fluoresces with equal or stronger intensity than is to be expected in the titration, set the upper scale reading of the meter with the "SET 100" control (Note 1).

e. With an empty compartment or a non-fluorescing solution set the lower scale reading with the "SET ZERO" control (Note 2).

f. If necessary, repeat steps D and E until the same readings are obtained (once is generally enough).

g. Place a magnetic stirring bar in the solution to be titrated, and adjust the mixing rate.

h. Titrate the solution by adding increments of titrant and reading fluorescence change on the output meter.

The entire setup and adjustments necessary for a titration can be made in a matter of minutes.

Note 1: On the initial setup of a new titration it may be necessary to adjust the reference beam intensity. This is done by closing the variable occluder until the meter needle jumps--this is caused by insuf-

ficient reference energy to drive the FET Q1 (see Figure 4)--and then opening it until the needle stabilizes. Additional slight adjustments can be made to maximize the sample signal.

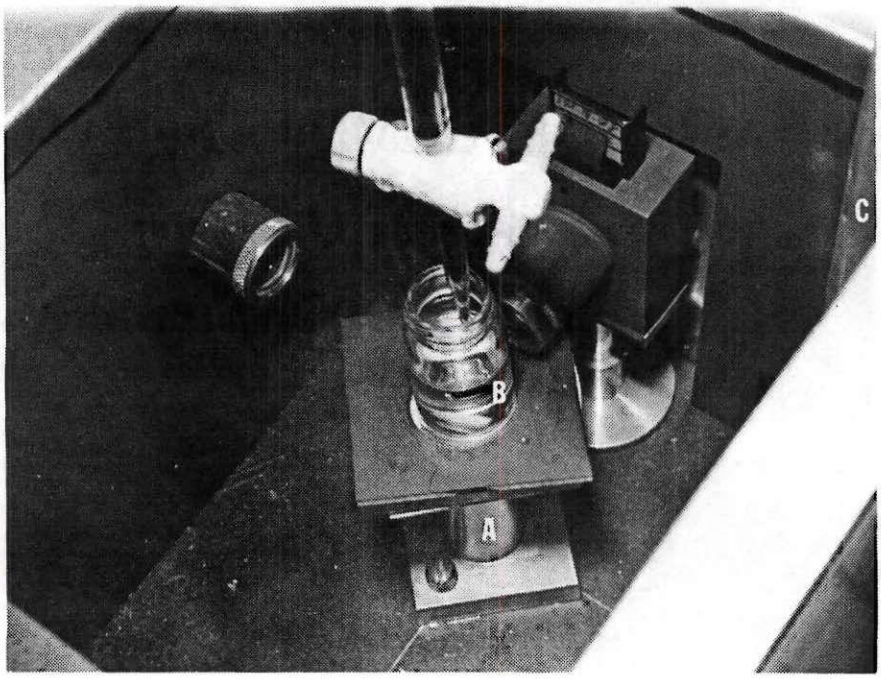
Note 2: Normally the span is not set at zero and 100 scale divisions but at 10 and 80 or 90 scale divisions. This allows a titration to be completed successfully even if the sample or blank contains an unexpected fluorescing impurity.

As was mentioned in the discussion of instrument operation, a micro-magnetic mixer is mounted in the sample compartment with the on-off and speed controls located on the front of the case. This device can provide mixing for sample container down to 1 cm in diameter and serves as base for most titration vessels. If another type vessel or mount is desired, the mixer can be removed by unplugging one electrical connection and removing one screw.

Finally, the size of the sample or titration area (18 x 20 cm) allows the operator a great deal of freedom in handling solutions and mounting special apparatus. A photograph of the sample compartment is shown in Figure 7 with a buret and titration vessel in position.

5. Sensitivity

In order to obtain a satisfactory level of sensitivity an 85-watt full range mercury lamp and a photomultiplier detector with an amplified output are used. In order to gain as much energy as possible from the exciting source relatively broad band pass filters are used. The sample radiation is detected by a photomultiplier. The signal from the photomultiplier goes through two amplification stages, first at the preamplifier and then at the sample integrater, before being displayed. The



A—micro-magnetic stirrer
B—titration vessel
C—rod mounting buret clamps or other equipment

Figure 7. Photograph of the Sample Compartment of the Fluorotitrator.

first amplification stage depends on the reference signal and can be increased by a decrease in the reference signal. The second amplification stage can give an additional 50-fold increase in signal and is controlled by the "SET 100" control.

CHAPTER III

PERFORMANCE EVALUATION

The fluorotitrator was tested with titrations that involved the increase and the decrease in fluorescent intensity.

A. Titration of Copper

The initial procedure attempted using the fluorotitrator was the titration of copper with EDTA to a calcein end point. In this titration the copper calcein complex is non-fluorescent but as the copper is complexed by the titrant the strongly fluorescing free calcein is released. The fluorescent intensity increases until all of the copper is complexed at which point the fluorescent intensity remains constant as titrant is added.

The primary filter employed has a peak transmittance at 480 nm and the secondary filter is a narrow band pass interference filter with a peak transmittance at 520 nm. The titration vessel employed in this series of titrations was a 20 ml scintillation tube.

Reagents:

1) Cu SO_4 - 0.025 F

2) EDTA - 0.025 F

3) Calcein - 10 mg/l in 1 F ammonia buffer pH 8.3

Procedure:

1) Add copper containing solution to the titration vessel (Note 1).

2) Adjust the volume to a total of 15 ml with buffer containing 0.1 mg/100 ml calcein.

3) Adjust the instrument to a reading of 80 with a solution containing only buffered calcein.

4) Insert the solution to be titrated. Start the magnetic stirrer and adjust the fluorotitrator to a reading of 10 (Note 2).

5) Titrate with the appropriate concentration of EDTA and read the fluorescence output of the solution after each increment of titrant (Note 3).

Note 1: The volume of copper containing solution to be added will depend, of course, on the concentrations of the copper and the EDTA used. In the titrations performed here in which equal concentrations were used 4 ml was the maximum volume which could be employed.

Note 2: Some care must be exercised not to allow the stirrer to run too fast. If the liquid vortex is in the exciting beam, errors result.

Note 3: At the outset of the titration the titrant can be added in fairly large increments since the instrument reading will not change from the original set point until the EDTA begins to displace the calcein from the copper-calcein complex. After the first positive change of more than three scale divisions occurs the succeeding increments of titrant should be much smaller.

A typical titration curve is shown in Figure 8. This curve was the first titration performed on the instrument and pertains to the titration of approximately 0.01 F copper with 0.01 F EDTA.

A series of titrations were performed on 0.5, 1, and 2 ml aliquots

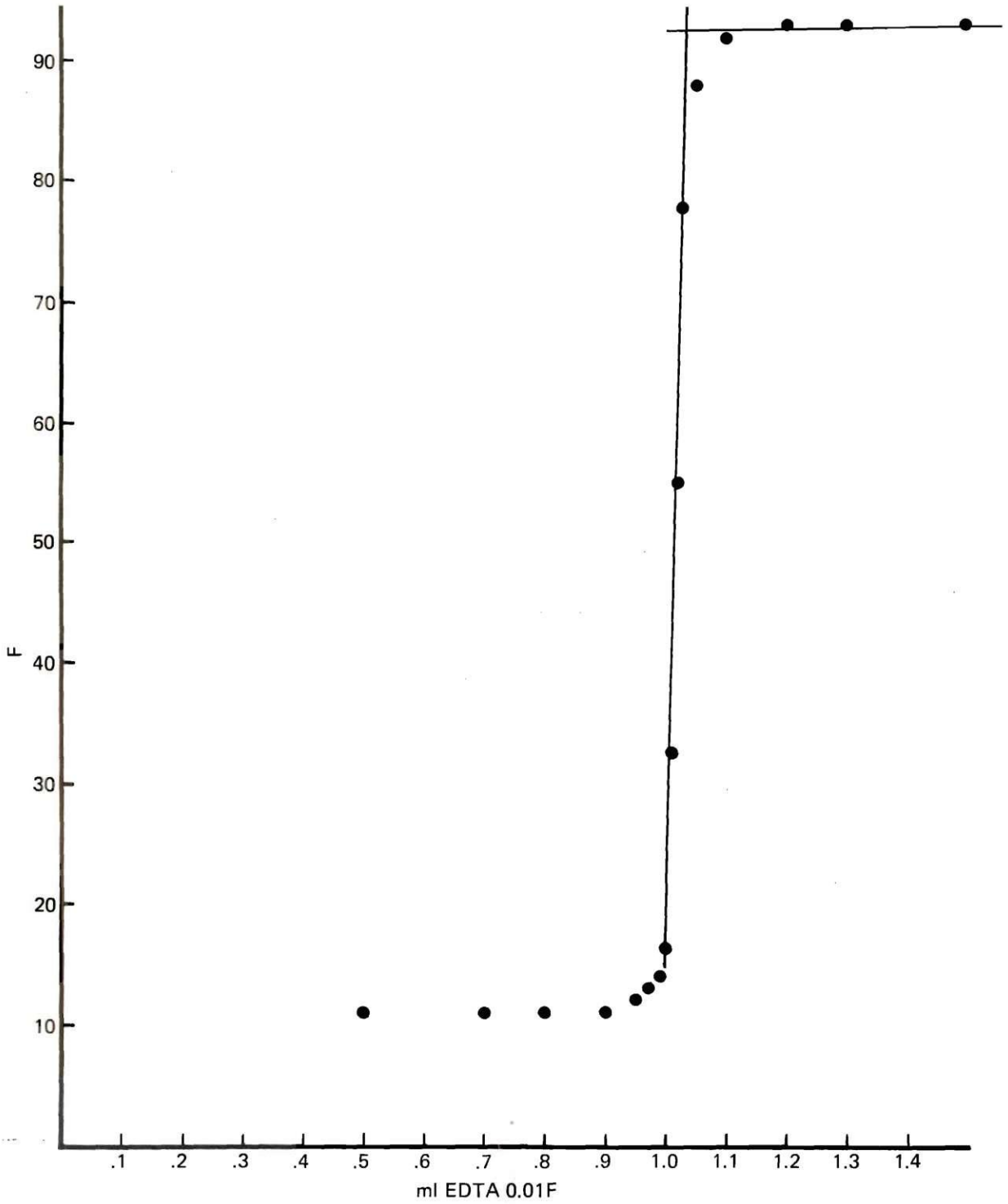


Figure 8. Titration Curve Involving the Appearance of Fluorescence Using the Fluorotitrator. (Titration of 0.01F cu. with 0.01F EDTA using calcein indicator.)

of 0.025 M copper using 0.025 F EDTA.

B. Titration of Calcium

The second type of titration used in the evaluation was one in which the decrease in fluorescent intensity was monitored. The procedure chosen was the titration of calcium with EDTA. Calcein was used as the fluoragenic agent. At a pH above 10 free calcein does not fluoresce but calcium forms a fluorescing complex. An excess of calcein was employed making the titration one of the self-indicating types. Thus, as the calcium is complexed by EDTA the fluorescence decreases.

In this titration the primary and secondary filters and the titration vessels were the same as those used in the copper titrations. Since one of the reasons for developing the fluorotitrator was to simplify certain analyses in biological fluids the concentrations of calcium used were 10 mg/100 ml which is approximately the same as that found in normal human serum. The sample size also was restricted to a maximum of 0.1 ml which is also compatible with a procedure for serum calcium. The operations involved in the assay are included in the following steps:

1. Pipet 0.1 ml of sample into the titration vessel and dilute to a final volume of 15 ml with 0.8F KOH which contains approximately 1 mg calcein/100 ml.

2. Using the "SET ZERO" control adjust the instrument to read 10 scale divisions with water in the light path.

3. With the "SET 100" control adjust the instrument to a reading of 80 scale divisions with the solution to be titrated in the light path.

4. Titrate with an appropriate concentration of EDTA. In this case the EDTA concentration was 2.3×10^{-4} F.

A typical titration curve is shown in Figure 9.

Table 1 shows results obtained on replicate titrations of the 10 mg/100 ml Ca samples. These titrations were performed over a two-day period and indicate that quite satisfactory precision can be obtained using the fluorotitrator.

Table 1. Results Obtained for Replicate Determinations of Calcium With the Fluorotitration

Ca Taken mg	ml 2.3×10^{-4} EDTA Used	Ca Found mg
.010	1.07	.0098
.010	1.08	.010
.010	1.07	.0098
.010	1.10	.012

The sample size can be reduced by using a smaller titration vessel. A vessel containing 5 ml of KOH diluent and .025 ml of sample is quite compatible with the instrument.

C. Linearity of Response

It can be seen in Figures 8 and 9 that the expected linear response is obtained as the concentration of the fluorescent species is increased or decreased. This shows that the instrument responds linearly within the concentration ranges encountered in the titrations performed. It was found that nonlinear response curves were generated as expected when concentrations of fluorescing solutions became too high. The difficulty was solved empirically and quickly by developing a fluorescence curve of fluorescence intensity vs. ml of reagent. The concentrations

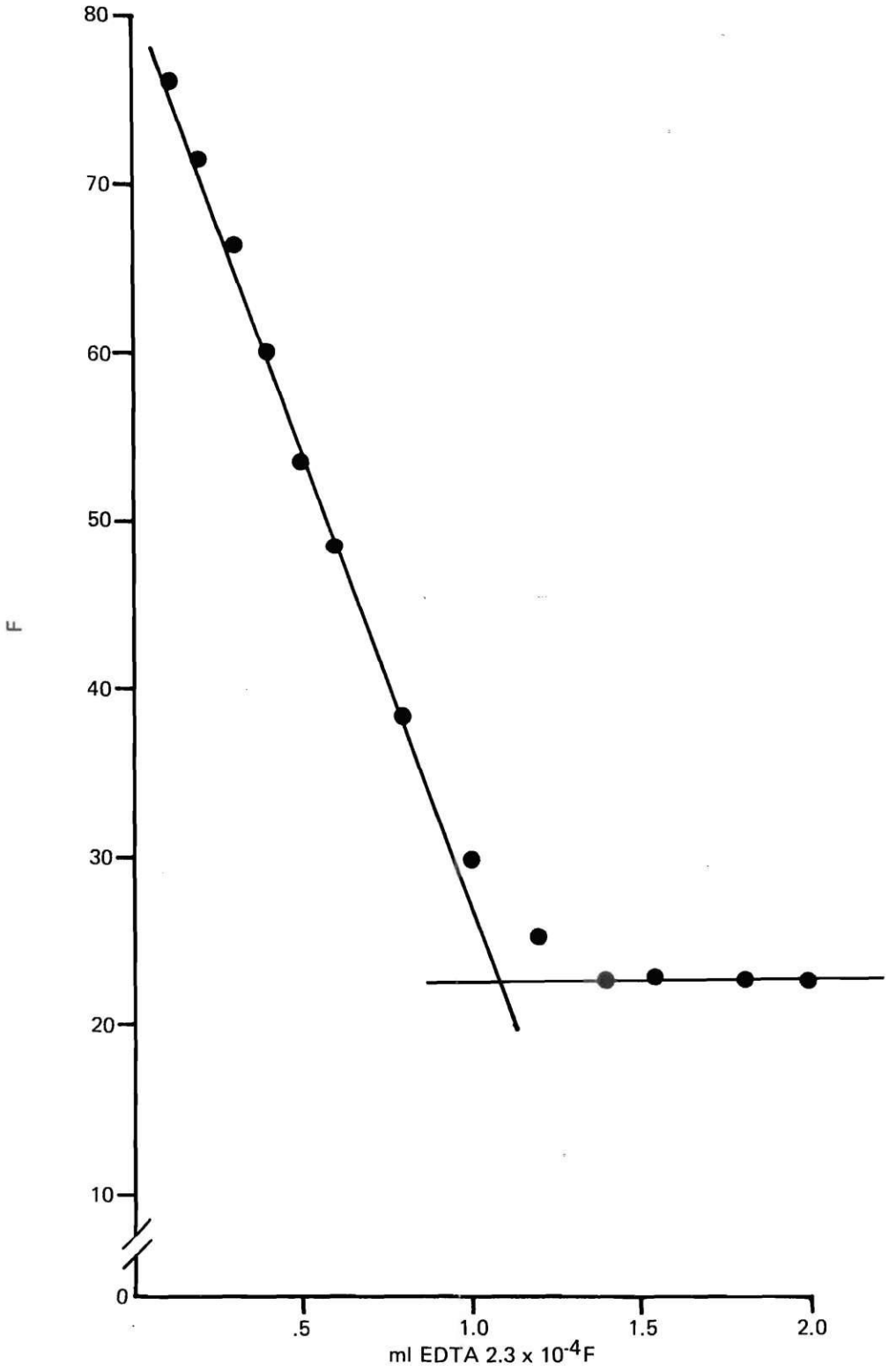


Figure 9. Titration Curve Involving the Disappearance of Fluorescence Using the Fluorotitrator. (Titration of 0.1 ml of 2.5 x 10^{-3}F Ca with 2.3 x 10^{-4}F EDTA in the presence of an excess of calcein to generate a linear titration.z)

were adjusted so that the titrations were performed within the region of linear fluorescence output.

D. Stability

The stability of the instrument was checked over various time spans at maximum sensitivity. Under these conditions the voltage applied to the photomultiplier tube was at the maximum (1500 v) and the optical occluder was closed as far as possible to drive the current-to-voltage converter A1 to its maximum gain. A typical result showed a drift of 6 scale divisions over a three hour period. Based on an initial scale setting of 80 this corresponded to a drift of approximately 2 per cent per hour. Since a titration is started and completed in a matter of minutes this drift is not significant. Also, such extreme gain adjustments are seldom necessary.

At the settings described above random fluctuation never exceeded 1% of full scale.

E. Convenience and Versatility

A number of qualitative tests have been performed on the instrument in checking limits of performance and possible applications. The sample container size for fluorescent measurements is limited at one extreme by the 3 mm x 8 mm dimensions of the exciting light beam and at the other extreme by the physical arrangement of the holder for the lenses E and E' and the collimating tube G (see Figure 2). The upper extreme, practically, is a 100 ml beaker but rectangular cells with even larger volumes could be accomodated. The lower extreme can be reduced by a reduction of the size of the incident beam. Measurements have been made

on a single drop of calcein solution suspended from a pipet. Various types of test tubes and disposable plastic vials were found to be readily accepted by the instrument. The plastic vials were not favored as titration vessels since most were found to be scratched or marred in packaging or shipping. Preliminary experiments also indicated that the instrument could be applied to nephelometric measurements as well as fluorescence measurements.

APPENDIX A

Relation of fluorescence to concentration.

a. $\frac{I}{I_o} = 10^{-abc}$ Beer's Law Relation

where:

I = Intensity transmitted

I_o = Incident intensity

a = absorptivity

b = optical pathlength through the solution

c = concentration

b. $1 - I/I_o = 1 - 10^{-abc}$ = fraction of light absorbed

c. $I_o - I = I_o(1 - 10^{-abc})$ = amount of light absorbed

The fluorescent intensity F is proportional to the quanta of light absorbed. ϕ = fluorescence efficiency (the ratio of quanta of light absorbed to quanta emitted).

d. $F = \phi K\ I_o(1-10^{-abc})$

10^{-abc} can be expanded - let $abc = x$

$$e^x = 1 + x + \frac{x^2}{2} + \frac{x^3}{6} + \cdots \frac{x^{n-1}}{n-1!} + \frac{x^n}{n!}$$

$$10 = e^{\ln 10} \text{ or } 10^x = e^{2.3\ x}$$

then

$$10^{-abc} = 1 + (-2.3abc) + \frac{(-2.3abc)^2}{2} + \cdots \frac{(-2.3abc)^n}{n!}$$

and

$$(1-10^{-abc}) = 1 - \left[1 + (-2.3abc) + \frac{(-2.3abc)^2}{2} + \cdots + \frac{(-2.3abc)^n}{n!}\right]$$

If the value for abc is less than 0.005 the latter terms in the expression can be dropped and

e. $F = k\phi I_o \cdot 2.3abc$

or

$F = K\ c$ where K includes the constants 2.3, a, b and ϕ.

REFERENCES

1. C. E. White and R. J. Argaver, Fluorescence Analysis, Marcel Dekker, Inc., New York, 1, (1970).

2. G. G. Rudolph, J. J. Holler, Jr. and W. J. Ford, Clin. Chim. Acta, 18, 187, (1967).

3. L. Meites, (Ed.) Handbook of Analytical Chemistry, McGraw-Hill Book Company, New York, 3, (1963).

4. B. Vecerek and O. Skovronsky, Chem. Listy, 47, 272, (1953).

5. R. L. Clements and G. A. Sergeant, Lab. Pract., 19, 813, (1970).

6. K. P. Stolyarov, N. N. Grigorev, and L. A. Soloveva, Zh. Analit. Khim., 17, 28, (1962).

7. H. K. Howerton and J. C. Wasilewski, Sym. on Titrimetric Methods, Ontario, Canada, 51, (1961).

8. K. Murakami and S. Kimura, Bunko Kenkyu, 6, 36, (1958).

9. A. B. Borle and F. N. Briggs, Anal. Chem., 40, 339, (1968).

10. R. L. Alexander, Clin. Chem., 17, 1171, (1971).

11. Corning Instruments Technical Bulletin.

PART TWO

PHOTOMETRIC TITRATIONS OF VANADIUM WITH 4-(2-PYRIDYLAZO)-RESORCINOL (PAR)

CHAPTER I

INTRODUCTION

A. General Remarks

The discovery of vanadium is attributed to Sefstrom of Sweden in 1830, who named the new element in honor of Vanadis, the Scandinavian goddess of love and beauty. The chemical behavior of the element was characterized primarily by Roscoe by 1870.

The first true industrial application of the element came with the discovery that when it was used as an alloying agent there was a marked improvement in the hardness of tool steels and armour plate. With the availability of an abundant supply of the vanadium from the Patron Mina Ragra deposit in Peru it was rapidly applied in the automotive, rail and tool-steel industries. Some of the other applications include use as an alloying agent in the titanium and aluminum industries and the use of vanadium pentoxide as a substitute for platinum in some catalytic processes (12).

While the industrially used vanadium comes from a relatively few deposits around the world, it is a common trace element in igneous rocks. It is estimated that vanadium is present to the extent of 0.15% of the earth's crust. The element is often found in small amounts in ores such as those of titanium, chromium, and iron, as well as in many petroleum crudes. The wide use and occurrence of this element, especially in trace levels, presents a need for a simple analytical method for the determina-

tion of vanadium which has a high degree of sensitivity and can be applied in virtually any laboratory.

B. Existing Methods

A large number of approaches have been utilized for the analysis of vanadium for various specific purposes. Among these approaches are pure instrumental techniques, spectrophotometric determinations, and redox and gravimetric analyses.

1. Instrumental Methods

Pure instrumental techniques include x-ray fluorescence, arc and spark emission spectroscopy, neutron activation, mass spectroscopy, and flame absorption spectroscopy (12). As a general statement these techniques offer a high degree of sensitivity and a greater selectivity for vanadium than the other analytical approaches. They are not without their drawbacks, however. The most significant of these is the limited availability of these expensive instruments to many laboratories. The one possible exception is the atomic absorption spectrophotometer. This technique, while perhaps the most generally used of all instrumental techniques for trace analysis, does not offer a high degree of sensitivity for vanadium. The "Standard Conditions" as published by Perkin-Elmer Corporation in 1973 (13) gives a detection limit of 1.7 μg/ml and suggests a practical working concentration of approximately 100 μg/ml. It is also reported that aluminum and titanium interfere.

2. Spectrophotometric Methods

Spectrophotometric determinations run a gamut of chromophores with varying degrees of sensitivity and practical usefulness (14-25).

In addition the tolerance for molybdenum in these techniques was relatively low, with the phosphotungstic acid method (14) reporting the highest tolerance for molybdenum, at a weight ratio of 200:1.

3. Photometric Titrations

The only photometric titration technique found in a review of the literature involved the titration of vanadium (V) with iron (II) in sulfuric acid solution. The absorbance is monitored at 375 nm or 760 nm the wavelengths of which iron (III) and vanadium (IV) absorb. Three modifications of this method were reported (26-28). None of these has an outstanding sensitivity and while the interference by molybdenum (VI) was not studied this metal can be expected to interfere seriously. In the modification described by Parab and Murthy (26) chromium (VI) is reported to interfere. The modification of Dikshitulu (27), however, permits the sequential titration of chromium (VI) and vanadium (V). No mention is given of interferences concerning the technique by Namiki (28).

There is much room for improvement in the direction of increasing sensitivity and freedom from interference especially from molybdenum.

CHAPTER II

PRELIMINARY INVESTIGATIONS

The following investigations were conducted in an effort to devise a procedure which would meet the criteria mentioned in the preceding section. Emphasis was placed on the development of a photometric titration from the outset. There were a number of reasons for this decision, the principal one being the fact that in photometric titrations greater amounts of foreign material can usually be tolerated than in photometric determinations. Any absorbance effects due to foreign material will offset a photometric determination. However, if absorbance changes are measured as in a photometric titration the analysis is often slightly effected if at all. Other reasons for selecting a photometric titration approach over a photometric determination lie in the advantages of a photometric titration over a photometric determination. These are exactly analogous to those discussed in Part One, concerning fluorometric titrations versus fluorometric determinations. Emphasis was placed on two areas. The area of primary concern was the development of a simple, sensitive, method for the determination of vanadium without regard for interferences. Such a method would be important because of the wide natural distribution of vanadium, often in matrices which do not offer interference problems. The second area of investigation was to enable vanadium to be determined in the presence of molybdenum.

A. Early Experiments

The initial investigations followed an approach similar to that described by Flaschka and Abdine (18). In this procedure vanadium (IV) is titrated with EDTA to a copper-PAN end point. These experiments were not productive for several reasons: (i) The experimental conditions suggested for the visual titrations were not suitable for application to a photometric titration; (ii) the sensitivity of the analysis proved to be limited and therefore did not offer promise as a method for the determination of trace amounts of vanadium; (iii) the conditions employed in the procedure cause the reduction of molybdenum (VI) also which then in its lower oxidation states is cotitrated.

A number of other approaches were investigated most of which for simplicity were initially tried as visual titrations; however none of them had the desired characteristics. One interesting approach involved a displacement reaction between vanadium (IV) and zinc in the zinc-EDTA complex. This technique proved promising for vanadium but only when present in relatively high concentrations. In addition, the reduced molybdenum species again were cotitrated.

B. The Application of PAR

The reagent 4-(2-pyridylazo)-resorcinol has been reported as a sensitive reagent for the colorimetric determination of vanadium (25,30-36). Preliminary investigations indicated the possibility of employing this reagent as the titrant in a self-indicating photometric titration of vanadium. In addition to forming a very intensly colored complex with vanadium (V) there was apparently no reaction with molybdenum (VI)

under the prevailing conditions. These factors promised to allow not only a very sensitive assay for vanadium but one which would be hopefully insensitive to large amounts of molybdenum.

C. Development of the Method

The reaction conditions necessary to utilize PAR as the titrant in a photometric titration were investigated.

1. Effect of pH

Initial experiments showed that the vanadium (V)-PAR reaction did not proceed as readily as would be necessary for a titration. A check of the pH effects on the reaction gave rise to the curve shown in Figure 10. The data for this curve were secured in the following manner: 10 ml of 1×10^{-4} F vanadium and 7 ml of 1×10^{-4} F PAR solutions were mixed, the pH was adjusted, and the volume brought to 50 ml. The absorption spectra of such solutions were obtained on a scanning spectrophotometer over the wavelength range between 400 and 700 nm.

A number of observations were made; (1) the absorbance maximum shifts with a change in pH, (2) for a given solution the absorbance increases with an increase in pH up to about pH 5.5, (3) at pH values above 5 the rate of formation of the vanadium-PAR complex is much slower than in the pH range between 4 and 5, (4) absorbance readings in the 415 nm region of the spectrum indicates that significant amounts of free PAR remained in the solutions at pH levels below 4 and above 6, even after long standing, and (5) the absorbance levels of the solutions remain constant even after 24 hours standing with no changes in wavelength of the absorbance maximum of a given solution. Most of the observations

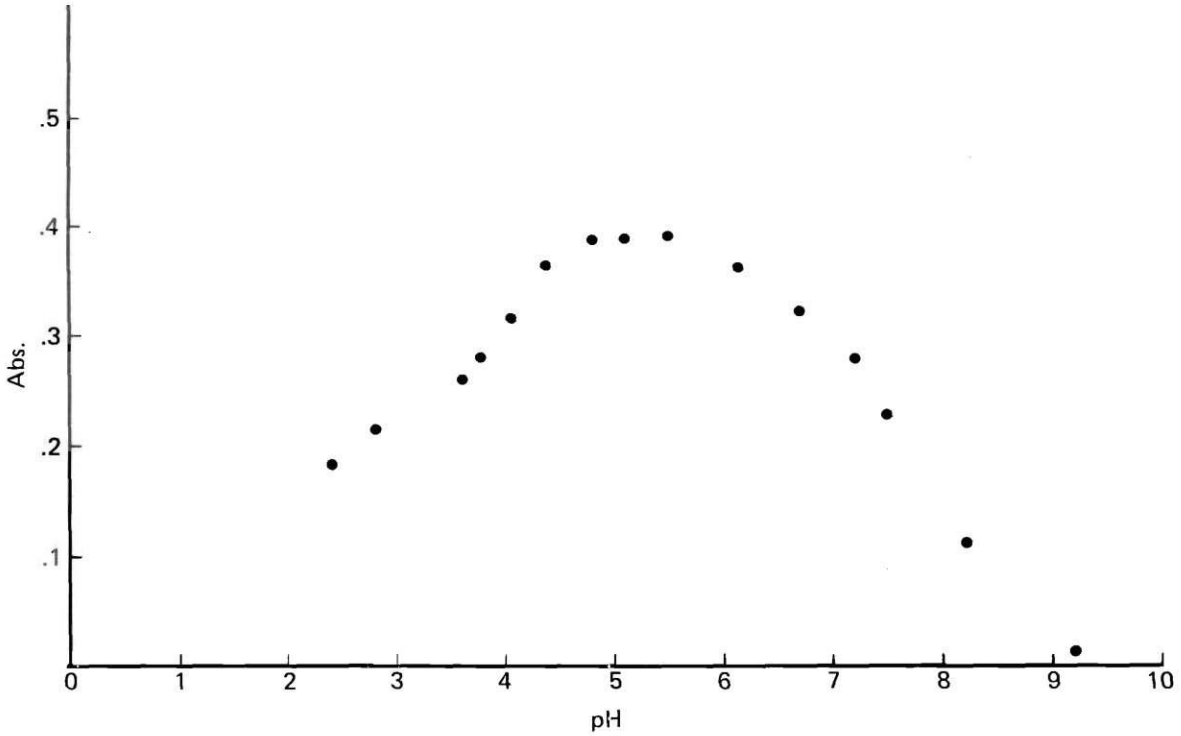


Figure 10. Effect of pH On the Vanadium-PAR Complex. (Absorbance measurements made at 542 nm.)

can be explained by the findings of Karpova et al. (37) who stated that PAR forms four different complexes with vanadium (V) in the pH region 1-8: $VORH^+$, VO_2RH, VOR, and VO_2R^-, where PAR is represented by RH_2.

Offhand, it would seem best to perform the titrations at a pH where the species VO_2R^- forms exclusively. This species has a reported molar absorptivity of 3.6×10^4 liters/mole-cm (37). Unfortunately, as was mentioned in the earlier observations, the formation of this complex takes place far too slowly and perhaps is interfered with too seriously by the formation of the VO_3^-. The presence of this species can be deduced from the findings that free PAR is present in the solutions at higher pH ranges. It was pointed out above that the composition of the solution was selected to contain an excess of vanadium. Therefore if a 1:1 complex is formed, which has sufficient stability to be used in a titration, no free PAR would normally be encountered.

Consequently, it was decided to concentrate on the pH region below 6 where the species VOR predominates. An acetate buffer of the pH range 4.5 to 5.0 was selected as the titration medium because of its high capacity and ease of preparation. Other buffer systems including phosphate buffer could be used in this pH range but investigation of these was deferred until a workable titration was developed. The rate of complex formation is high and the absorptivity although somewhat less than that mentioned above is still sufficiently high at 3.3×10^4 liters/mole-cm.

The final observation made in this experiment indicates that once a particular species (or combination of species) has formed, there is no change in the makeup of the solution with time. This gives hope that accurate titrimetric analyses would be possible even though more than one

complex is formed.

2. Wavelength Selection

The absorbance spectra of the vanadium-PAR complexes and of free PAR are shown in Figure 11. The magenta vanadium-PAR complex VOR shows a single absorbance maximum in the visible spectral range at 542 nm. The measured absorptivity under the conditions selected is 2.9×10^4 liters/mole-cm which is slightly lower than the value of 3.3×10^4 reported by Karpova and associates (37). As can be seen from Figure 11, there is practically no interference from free PAR at the wavelength maximum of the vanadium-PAR complex; therefore, this wavelength was selected as appropriate for the photometric titration of vanadium (V) with PAR.

D. Vanadium in the Presence of Molybdenum

As has been mentioned earlier, the chemical similarities between vanadium and molybdenum tend to make the separation of these elements difficult. The problem is compounded when one is interested in trace amounts of vanadium. In such a case the risk of contamination or loss of material is considerably increased when the incorporation of a separation increases the number of steps.

The initial observations that molybdenum (VI) does not form a colored complex with PAR could be taken to imply that vanadium could be readily determined in the presence of molybdenum. When titrations were performed in the presence of molybdenum (VI) it was found, however, that as the molybdenum concentration increases, the rate of the vanadium-PAR color formation decreases and the shape of the titration curve deterio-

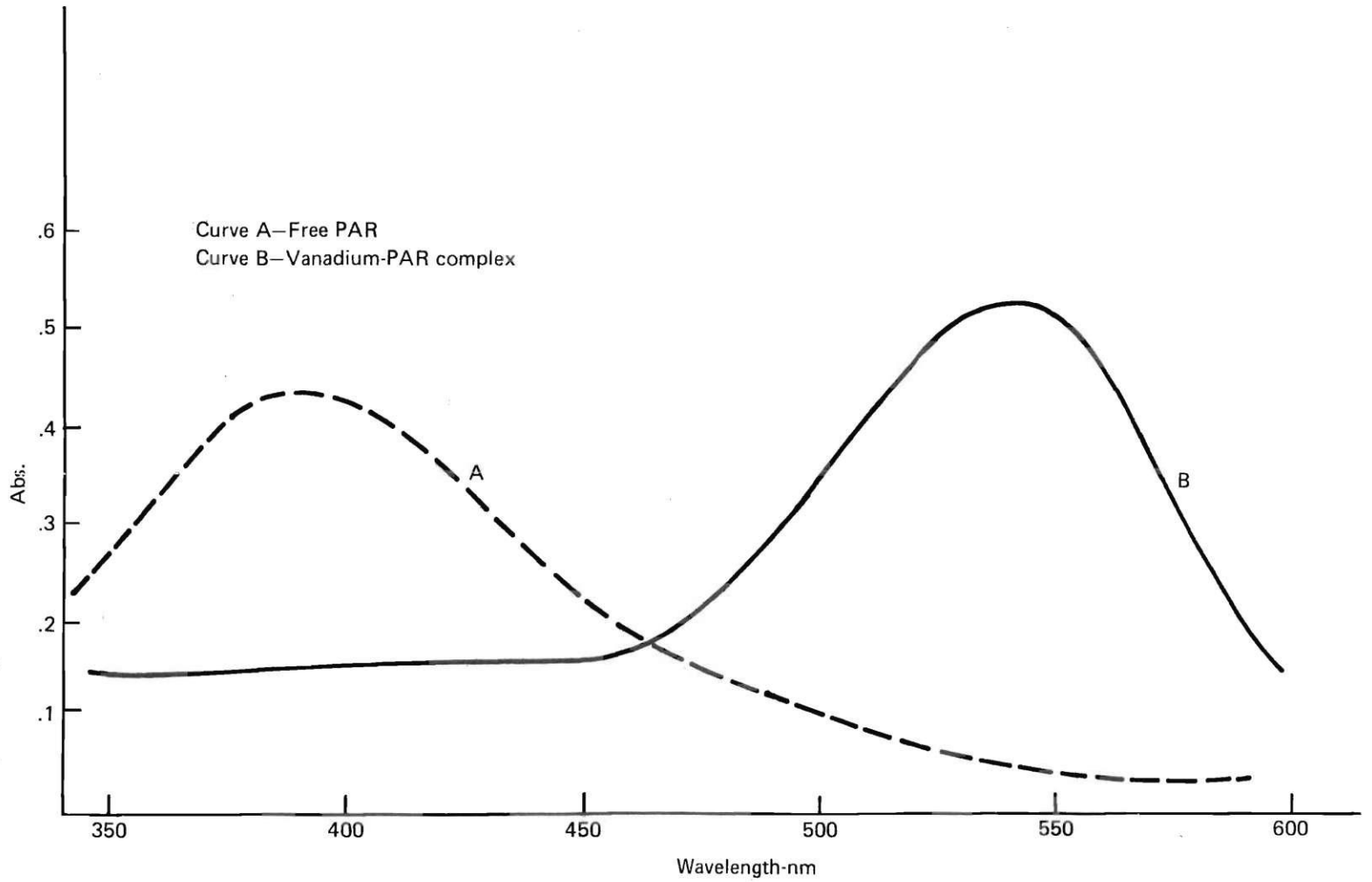


Figure 11. Absorption Spectra of Free PAR and the Vanadium-PAR Complex.

rates.

The most reasonable explanation for this situation is the formation of heteropoly acids between molybdates and vanadates. It can readily be concluded from equilibrium considerations that with increasing concentrations of molybdenum more and more of the vanadium will be tied up as the heteropoly acid and will be unavailable for the formation of the PAR complex.

It was found that under the conditions selected for the titration of vanadium alone a threshold value for molybdenum existed below which vanadium could be titrated without adverse effects. This threshold value is a molar ratio of 300:1 of molybdenum over vanadium. This corresponds to a weight ratio of 570:1. While this tolerance level for molybdenum marks a significant improvement over most techniques which have been proposed for the determination of vanadium, a number of investigations were carried out in an effort to increase the allowable excess still further.

1. Temperature

Since the effect of molybdenum is to decrease the rate of reaction of vanadium with PAR the most obvious first approach was to increase the temperature of the reaction mixture. A temperature controlling system was devised for use with the phototitrator which would heat the reaction mixture to a desired level and hold the temperature to within $\pm 0.2^{o}C$. Using this device, titrations were performed at temperatures up to $40^{o}C$. At higher temperatures bubble formation on the probe of the semi-immersion phototitrator became so pronounced that titrations were not feasible. In order to evaluate the effect of even higher temperatures solutions were

heated externally to the phototitrator, allowed to cool and returned to the instrument for measurement. These efforts proved fruitless and all succeeding titrations were performed at room temperature.

2. Masking

Since the tolerance level for molybdenum was not increased by non-chemical techniques, attention was then turned toward reducing or eliminating the effect of molybdenum by a masking technique. Hopefully, a compound could be found which would preferentially react with molybdenum and leave vanadium free to react with PAR. Toward this end a rather extensive group of reagents was tried. Included in the reagents tested were:

Succinate
d-Mannitol
Tartrate
Nitrilotriacetic acid
N-Hydroxyethylethylenediaminetriacetic acid
Ethylenediaminepentaacetic acid
Ethylenediaminedi(o-hydroxyphenylacetic acid)
Pyrocatechol
Dihydroxyglucinate
Citrate
Oxalate
Sulfosalicylate
Ethylenediaminetetraacetic acid
Quardol N,N,N',N', tetrakis(2-hydroxypropyl)ethylenediamine
1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
Glycerol
Ethylene glycol

As might be expected, due to the similarities of the two compounds of interest it was found that both vanadium and molybdenum reacted with most of the compounds tested in a similar fashion. A few compounds showed promise; included among these were oxalate, citrate, succinate, EDTA, EGTA and DCTA. Of this group EDTA proved to be most useful. When a succinate buffer was used in place of acetate the rate of color forma-

tion was enhanced slightly; however, this improvement did not provide a sufficient reason to replace the acetate buffer which has a higher buffering capacity. When an excess of either of the compounds other than succinate was used, as is generally done in masking procedures, it was found that titrations were disrupted.

In view of the fact that a relatively high level of molybdenum can be tolerated without an appreciable effect on the titration of vanadium it was decided to employ the technique of substoichiometric masking. This technique is explained in detail by Flaschka and Garrett (38,39).

CHAPTER III

EQUIPMENT AND CHEMICALS

A. Instruments

The bulk of the titrations were performed on a phototitrator; however, other photometric measuring instruments were used for comparative titrations and for investigations of absorbance characteristics of the vanadium (V)-PAR complex.

1. Spectrophotometers

Absorbance curves were obtained with either a Bausch and Lomb Spectronic 505 or a Beckman DB spectrophotometer. Photometric titrations were performed on the Beckman DB and a Cary Model 16 spectrophotometer. The Cary Model 16 was accepted as the reference instrument for definition of absorbance values and for determining whether or not a system adhered to Beer's law.

2. Phototitrator

The Flaschka-Butcher phototitrator (40) was the only instrument of this type used. Some problems were encountered with the application of this instrument in early titrations. A considerable non-linearity was noted almost from the onset of the titration. When linearity was checked on the Cary 16 spectrophotometer, it was found that the solutions involved showed good adherence to Beer's law up to an absorbance of at least 1.0. The addition of a heat absorbing filter to the interference filter in the phototitrator provided a significant improvement in linear-

ity, thus indicating the fault to be with the instrument. Long wavelength stray light passes through the monochromating filter and reaches the detector. The photodiode used as the detector is extremely sensitive in the long wavelength region and thus severe non-monochromacy is created. The problem was fully eliminated by using interference filters that are blocked to infinity on the long wavelength side.

3. pH Meters

All pH measurements, except one set, were made on a Corning Model 7 pH meter. The set of measurements excepted involved titrations at various pH levels and a Corning Model 12 pH meter was used because of its higher level of stability and accuracy. The pH meters were standardized with Beckman standard buffers at either pH 4.01, 7.00, or 9.18 as appropriate for the measurements needed.

4. Glassware

The usual glassware such as beakers, flasks, etc., were used as needed. For volumetric measurements Class A volumetric glassware was used exclusively and without additional calibration.

Glassware washing procedures generally involved a detergent wash and rinse with tap water, then a final rinse with deionized distilled water. The final rinse was repeated five times with a minimum volume equal to 10% of the container volume.

B. Reagents and Solutions

1. All water used in the titrations and in preparing and diluting reagents and solutions was either doubly deionized or distilled and deionized.

2. All reagents used met ACS analytical reagent specifications. In every instance possible, Baker Analyzed Reagents were used, and without further purification. The following list includes the major reagents used and the method of preparing their solutions. A number of metal salts as well as chelating agents were tested in various phases of the investigations. The solutions of these reagents were prepared by standard techniques and they played no role in the final procedure.

a. Ammonium Meta Vanadate

All solutions for test titrations were prepared for this reagent. Generally two concentration levels were prepared.

Vanadium Stock Solution, 1.000×10^{-2} F. Weigh 1.1699 grams of ammonium meta vanadate and place into solution in about 500 to 700 ml of water. Gentle warming may be necessary. Cool to room temperature, transfer to a 1000-ml volumetric flask, and dilute to mark with water.

Vanadium Working Solution, 1.00×10^{-4} F. Transfer 1.00 ml vanadium stock solution to a 100-ml volumetric flask and dilute to mark with water. This solution is used to define the concentration of the PAR titrant and should be prepared fresh weekly or as needed.

b. PAR

The quality and apparent purity of the PAR used was found to vary depending upon the source of the reagent. Some efforts were made at reprecipitation and drying of the material on hand. It was found that no great improvement was achieved. The stability of the prepared solutions of PAR is excellent. Therefore, it was decided to standardize the PAR titrant solutions by titration of standard vanadium solution.

PAR Stock Solution, 0.01 F. Weigh 0.5 g of PAR, slurry with

approximately 50 ml H_2O and add dropwise dilute 0.01 F NaOH to achieve dissolution. Transfer to a 200-ml volumetric flask and dilute to mark with water. Stable indefinitely.

PAR Working Solution, 5×10^{-4} F. Dilute the PAR stock solution 1:20 with water.

c. Sodium Molybdate

This salt was used for checking molybdate interferences. Prepare 0.1 F stock solution by weighing 24 g of the salt, placing it into a 1000-ml volumetric flask and diluting to the mark with water.

d. EDTA, 0.1 F

Dissolve 37.2 grams of the disodium salt of EDTA in water, add a few pellets of sodium hydroxide to hasten dissolution, dilute to mark in a 1000-ml flask. From this stock solution, prepare more dilute solutions as needed.

e. Acetate Buffer, pH 4.8, 1 F.

Dissolve 82 g sodium acetate trihydrate in 800 ml water, adjust the pH with HCl, and bring to 1000-ml with water.

CHAPTER IV

TITRATIONS

A. The Determination of Vanadium Alone

1. Procedure

a. Adjust the pH of the solution to between 4.5 and 5.0.

b. Add 5 ml of 1 F acetate buffer pH 4.8.

c. Adjust the volume to a convenient known total volume (Note 1).

d. Adjust the wavelength to 542 nm, and the transmittance to 100% with the initial solution in the light path.

e. Add a small increment of standardized PAR solution and record the absorbance reading (Note 2).

f. Continue the addition of increments of PAR and absorbance measurements until several (3-5) readings are obtained that are essentially identical.

g. Plot measured absorbance versus milliliters of titrant added. Determine the endpoint as the intersection of the straight line segments generated during the initial and final phases of the titration (Note 3).

Note 1: The final volume will depend on the concentration of vanadium. Final solution concentrations between 0.5 and 1.5 μg/ml provide satisfactory absorbance readings with a 1-cm light path. Total volumes of from 20 to 200 ml can be handled conveniently.

Note 2: The size of the increments of titrant should be quite small in the initial stages of the titration. In general it is pref-

erable to define the initial line segment by the time the titration is half way to the endpoint. If the analyst has no indication of the concentration level to be titrated quick pretitration or screen is advisable. After the half way point the increments of titrant can be increased or after some experience with the system the analyst can make several initial readings and can then proceed directly to several readings well past the endpoint.

Note 3: If a conventional spectrophotometer is used to monitor the progress of the titration a portion of the solution being titrated must be transferred from the titration vessel (usually a beaker) to a cuvet after the addition of each increment of titrant and returned to the titration vessel after the absorbance is measured. The additional steps do not significantly detract from the titration as long as none of the solution is lost in the transfer operations. The precaution of placing a very light film of grease beneath the pouring spout of the beaker and around the top edge of the cuvet will prevent drop loss.

Note 4: The endpoint can be determined by either plotting the points and drawing the best fit straight lines as is shown in Figure 2 or by calculating the stopes and intercepts of the lines by least squares approximations. The two line segments are $y = a_1x + b_1$ and $y = a_2x + b_2$. At the point of intersection $a_1x + b_1 = a_2x + b_2$ and $x = \frac{(b_2 - b_1)}{(a_1 - a_2)}$.

2 Results

The following sections present some of the results obtained from the titrations of vanadium in the absence of interfering compounds.

Tables 2 through 4 show data obtained with three different instruments. The precision, as indicated by the standard deviation, is about

Table 2. Titrations of Vanadium Using a Beckman DB-G Spectrophotometer

Data collected over a 3-week period. Two different PAR solutions were used.

μg V Taken	μg V Found	Δμg[a]	% Error
50.95	50.33	-0.62	1.12
	50.52	-0.43	0.84
	50.44	-0.51	1.00
	52.22	+1.27	2.49
	51.23	+0.28	0.55
	50.78	-0.17	0.33
	51.66	+0.71	1.39
	50.78	-0.17	0.33
	50.89	-0.06	0.12

Mean Error = 0.92%

$\bar{x}$ = 50.98

SD = $\pm$0.62

Lower Confidence Limit = 50.57

Upper Confidence Limit = 51.39

Lower Maximum Percent Bias = -0.8

Upper Maximum Percent Bias = +0.8

a Δ = (μg Found - μg Taken)

Table 3. Photometric Titrations of Vanadium Using a Semi-Immersion Phototitrator

Data collected over a 2-month period.

μg V Taken	μg V Found	Δμg[a]	% Error
51.46	51.83	+0.37	0.72
	51.26	-0.20	0.39
	51.41	-0.05	0.12
	50.97	-0.49	0.95
	52.12	+0.66	1.28
	51.26	-0.20	0.39
	51.26	-0.20	0.39
	52.70	+1.24	2.41
	51.83	+0.37	0.72
	51.26	-0.20	0.39
	50.68	-0.78	1.52
	50.39	-0.07	0.14
	50.97	-0.49	0.95

$\bar{x}$ = 51.46

SD = $\pm$0.68

Upper Confidence Limit = 51.83

Lower Confidence Limit = 51.08

Upper Maximum Percent Bias = 0.7

Lower Maximum Percent Bias = -0.7

a. Δ = (μg Found - μg Taken)

Table 4. The Photometric Titration of Vanadium Using a Cary 16 Spectrophotometer

Data collected over a 1-month period. Two concentrations of PAR were used.

μg V Taken	μg V Found	Δμg[a]	% Error
50.54	49.92	-0.62	1.23
	49.83	-0.71	1.40
	49.81	-0.73	1.44
	51.01	+0.47	0.93
	50.79	+0.25	0.49
	50.62	+0.08	0.16
	50.55	+0.01	0.02
	50.97	+0.43	0.80
	51.45	-0.09	0.18
	51.79	+0.25	0.49
	51.27	+0.73	1.44
	51.79	+1.25	2.47
	50.76	+0.22	0.44
	49.71	-0.83	1.64
	50.43	-0.11	0.22
	50.76	+0.22	0.44

$\bar{x}$ = 50.65

SD = 0.62

Upper Confidence Limit = 50.97

Lower Confidence Limit = 50.32

Upper Maximum Percent Bias = 0.8

Lower Maximum Percent Bias = -0.4

a. Δ = (μg Found - μg Taken)

the same for each set of data. This indicates that equally good results can be obtained whether the titration is performed outside of the measuring instrument and transferred to the cuvet or is performed in the cell of a specially designed phototitrator. The precision of the data in these tables are meaningful indicators of the reliability of the titration since the data were collected over extended time periods and thus contain elements of variation which would be hidden in simple replicate assays.

The data in the tables allow an estimate of the accuracy. Based on the number of determinations listed it is possible to calculate upper and lower confidence limits. This says that one can state with 95% certainty that the mean value of an infinite number of assays will fall within these limits. When this range is compared to the "true" value, in this case the amount of vanadium taken, it can be seen that the "true" value lies within the limits and very close to the mean value. This comparison is given in the tables in the form of the upper and lower percent bias where the difference between the "true" value and each of the limits is divided by the "true" value.

Table 5 shows the results obtained when titrations were performed on a number of concentration levels of vanadium ranging from 0.5 μg/ml to 2 μg/ml. These results are presented in terms of total amounts found.

A least squares analysis of these data gives a correlation coefficient between the "true" values and the observed results of 0.9999 (1.00).

The concentration range shown is about optimal for this procedure. Amounts of vanadium above 100 μg can be brought into this concentration

Table 5. Results Obtained from Titrations of Vanadium at Various Concentration Levels

μg Taken	μg Found	$\Delta\mu g^a$	% Error
25.7_4	25.6	-0.1_4	0.5
30.5_7	30.6	$+0.0_3$	0.1
35.6_7	35.6	-0.0_7	0.1
40.7_6	41.2	$+0.4_4$	1.1
45.8_6	46.0	$+0.1_4$	0.3
50.9_5	50.9	-0.0_5	0.1
101.9	103.1	+1.2	1.1

Least Squares Regression
Slope = 1.01_7
Correlation Coefficient = .9999

a. Δ = (μg Found - μg Taken)

range simply by; increasing the total final volume of the solution and by increasing the concentration of the titrant. Smaller amounts of vanadium can be handled by reducing the final volume; for example, a final volume of 25 ml is easily achievable and will allow titrations of less than 10 μg of vanadium. If a phototitrator or versatile spectrophotometer is used the path length can be adjusted to achieve the same results. The concentration of titrant again should be adjusted in order to be able to measure the amount of titrant added accurately. All titrations in these studies were performed using either a 5 or 10 ml buret. If a micro buret is available then even more options are open as far as sample size and titrant concentrations are concerned.

The effect of pH on the reaction between vanadium and PAR has already been noted. While this can cause particular difficulties in direct spectrophotometric determinations it was found that titrations could be accomplished successfully over a rather wide pH range as long as the pH remained the same during the course of the particular titration being performed. The results of a series of titrations at different pH levels are given in Table 6. Three of the titration curves are shown in Figure 12 to demonstrate that although there are significant changes in initial slopes and final absorbance values, the endpoints are the same and are quite easily located. As was expected from the early investigations at pH levels above 5.2 the reaction begins to drag noticeably. At pH 5.8 the rate of color formation is so slow that a titration requires in excess of one hour to be completed. For this experiment each solution was prepared by mixing 10 ml of 1.00×10^{-4} F vanadium salt with 5 ml of acetate buffer pH 4.7. Then the pH was ad-

Table 6. Effect of pH on Titration of V(v) with PAR

μg V Taken	μg V Found	pH Start	pH End
50.9_5	50.9	3.6_8	3.7_1
	50.7	4.1_0	4.1_3
	51.3	4.2_6	4.3
	50.9	4.6_7	-
	51.3	5.0_4	5.0_7
	51.3	5.4_3	5.5
	52.6	5.8_1	5.9_3
	53.6	6.2_2	6.3_4
	-	6.6	7.9_2

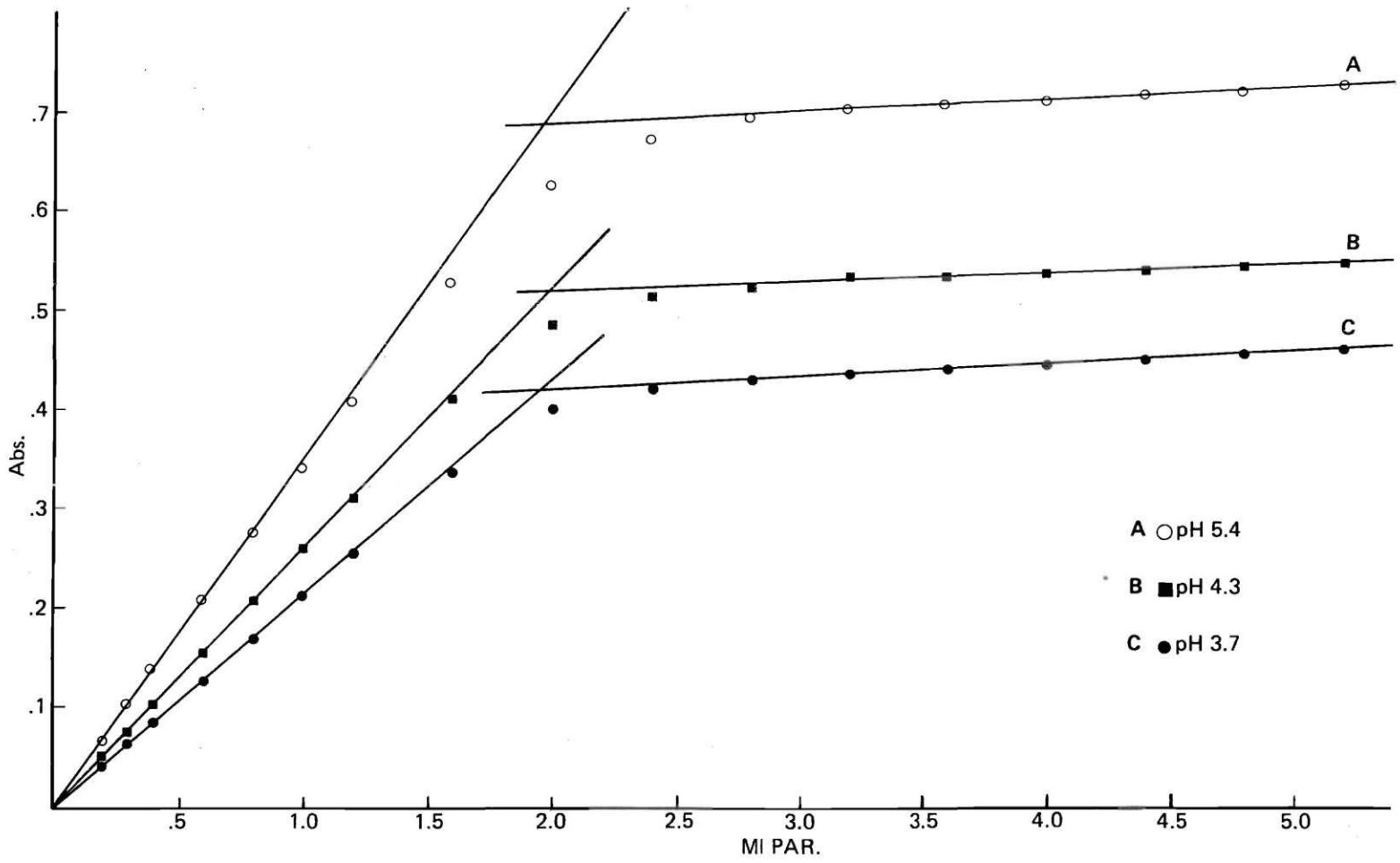


Figure 12. Photometric Titration Curves of Vanadium With PAR at Different pH Levels.

justed and the solution brought to a final volume of 50 ml with distilled deionized water and titrated with PAR. The pH of the solution was measured a second time immediately after the completion of the titration. The inability to obtain a result at pH 6.6 is attributed to the rather large change in pH during the course of the titration.

B. The Determination of Vanadium in the Presence of Molybdenum

1. Procedure

The procedure used for the determination of vanadium in the presence of up to a 600 times excess of molybdenum is exactly the same as for vanadium alone. If the excess of molybdenum is more than 600:1 the following steps are to be taken:

a. Adjust the pH of the solution to between 4.5 and 5.0

b. Add sufficient EDTA to complex all but an approximately 200 times excess of molybdenum (Note 1).

c. Add 5 ml of 1 F acetate buffer pH 4.8.

d. Adjust wavelength and titrate as described in the procedure for vanadium alone.

Note: One technique was found useful for the sub-stoichiometric masking of molybdenum. The solution is prepared exactly as done for vanadium alone up through the step of the addition of acetate buffer. At this point instead of diluting the sample add the first increment of PAR. Then add from a buret EDTA of an appropriate concentration until the absorbance of the solution is a maximum. Next, add to the solution molybdenum (VI) until it is in excess of the vanadium by 100 to 300 times. Finally, dilute the solution to an appropriate known volume and

titrate.

2. Results

Some results obtained for titrations of vanadium in the presence of excesses of molybdenum are shown in Table 7. It might be noted that there is some effect of molybdenum shown as the excess becomes very large and the limit of usefulness appears to lie at an excess of molybdenum over vanadium of about 19,000:1.

Table 7. Determinations of Vanadium in the Presence of Various Amounts of Molybdenum

μg V Taken	μg V Found	Δ[a]	% Error	Ratio of Molybdenum to Vanadium	Masking Agent
51.4	51.2	-0.2	0.4	380:1	None
	51.6	+0.2	0.4	570:1	None
	50.9	-0.5	1.0	940:1	EDTA
	52.6	+1.2	2.3	1880:1	EDTA
	53.4	+2.0	3.9	1880:1	EDTA
	54.5	+3.1	6.0	4700:1	EDTA
	55.6	+4.2	8.1	18,800:1	EDTA

a. Δ = (μg Found - μg Taken)

CHAPTER V

INTERFERENCES

As might be expected a ligand such as PAR forms complexes quite readily with a number of cationic species, some of these are iron, lead, copper, cobalt, magnesium, and manganese. Any of these species when present in sufficient excess will make the titrimetric endpoint for vanadium difficult if not impossible to locate. The best solution is to use one of several techniques which will allow the separation of molybdenum and vanadium from these species (41).

Special consideration was paid to chromium (VI) and tungsten (VI). In the case of chromium there is no interference with the reaction itself; however, the yellow color of the chromate solutions does create a background which if the concentration is too high may cause problems with absorbance measurements on simple instruments. Titrations were performed in the presence of a 2500:1 ratio of chromium to vanadium with no difficulty at all.

Unfortunately tungsten (VI) and any degree of excess ties up the vanadium so that reaction with PAR is blocked. However, tungsten is easily separated from vanadium and this seems to be the best solution to the problem.

Common anionic species such as chloride, nitrate, and sulfate cause no difficulties even at concentrations of several moles per liter. This fact indicates that most of the popular opening procedures can be

used with the proposed titrimetric finish. Citrate and oxalate when present in excess tend to inhibit color formation; however, when these species were applied in substoichiometric amounts considerable improvements were obtained in titrations of vanadium in the presence of up to approximately 5,000 times excess of molybdenum.

When phosphate was introduced to the system it was found that a positive bias was observed in the titrations of vanadium. This bias increased with increasing concentrations of phosphate and was sufficient to eliminate the use of phosphate buffer and not to recommend its use for the titration described.

REFERENCES

12. Treatise on Analytical Chemistry, I. M. Kolthoff and P. J. Elving Ed. Part II, Vol. 8, Interscience Publishers, New York, 239, (1963).

13. Perkin Elmer Bulletin, Analytical Methods for Atomic Absorption Spectrophotometry, Standard Conditions for Vanadium, (1973).

14. E. B. Sandell, Colorimetric Determination of Traces of Metals, Interscience Publishers, New York, 607, (1950).

15. S. Nishikawa, Y. Nakagawa, M. Satake, and T. Matsumoto, Bunseki Kagaku, 15 (9), 944, (1966).

16. A. I. Buseu and Z. P. Karyakina, Zh. Anal. Khim., 22 (10), 1506, (1967).

17. V. B. Badri, and A. Dey, Mickrochim Acta, 3, 664, (1969).

18. R. Jakubiec, and D. Boltz, Anal. Chem., 40 (2), 446, (1968).

19. S. Z. Nguyen, V. L. Ryzhenko and F. G. Zarovskii, Ukr. Khim. Zh., 35 (2), 206, (1969).

20. K. C. Srivastava, S. Banerji, Chim. Anal., 52, (9), 973, (1970).

21. A. K. Majumdar, S. K. Bhowal, Analyst, 96, 127, (1971).

22. W. Pilz, S. Komischke, and G. Prior, Int. J. Environ. Anal. Chem., 1 (1), 47, (1971).

23. V. N. Podchainova and V. Ya. Dergachav, Tr. Ural. Politekh. Inst., No. 148, 112, (1966).

24. R. S. Tramm, K. S. Pevzener, R. A. Kushnareva, Khim. Metody Anal. Niobiya Splavov Ego Osn., 36, (1970).

25. B. V. Agarwala and A. K. Dey, Microchem. J., 12 (2), 162, (1967).

26. S. V. Parab, T.K.S. Murthy, India, A.E.C., Bhabha At. Res. Cent., 611, (1972).

27. L. S. A. Dikshitulu, Ind. J. Chem., 9 (8), 872, (1971).

28. H. Namiki, Bunseki Kagaku, 13 (4), 354, (1964).

29. H. A. Flaschka and H. Abdine, Chemist-Analyst, 45, 58, (1956).

30. A. Calvo, Y. Calvo and N. Maggio, Bol. Soc. Chilena Quim., 13 (1) 9, (1963).

31. S. P. Mushran, O. Prakash, and J. R. Verma, Bull. Chem. Soc. Jap., 45 (6), 1709, (1972).

32. T. Yotsuyangi, J. Itoh, and K. Aomura, Mem. Fac. Eng., Hokkaido Univ., 13 (1), 39, (1971).

33. T. Yotsuyangi, J. Itoh, and K. Aomura, Talanta, 16 (12), 1611, (1969).

34. O. Budevski, and L. Dzhonova, Talanta, 12 (3), 291, (1965).

35. W. J. Geary, and C. N. Larsson, Proc. SAC (Soc. Anal. Chem.) Conf. Nottingham Engl., 455, (1965).

36. M. Kawahata, H. Mochizuki, R. Kajiyama, and K. Ichihashi, Bunseki Kagaku, 14 (4), 348, (1965).

37. O. I. Karpova, V. V. Likachina, and A. T. Pilipenko, Ukr. Khim. Zh., 39 (2), 194, (1973).

38. H. Flaschka and J. Garrett, Talanta, 15, 589, (1968).

39. H. Flaschka and J. Garrett, Talanta, 15, 595, (1968).

40. H. Flaschka and J. Butcher, Talanta, 12, 913, (1965).

41. Treatise on Analytical Chemistry, Op. Cit., 203, (1963).

PART THREE

TWO AUTOMATED PROCEDURES FOR THE DETERMINATION OF SERUM IRON AND IRON BINDING CAPACITY WITH THE COLOR REAGENT FERROZINE

CHAPTER I

INTRODUCTION

A. General Remarks

Continued interest in the application of photometric titration technique for the analysis of trace elements caused attention to be drawn to a new reagent. This compound, (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic-acid)-1,2,4-triazine) is known by the trivial name ferrozine and was introduced by Stookey (42) as a reagent for the photometric determination of iron.

B. Properties of Ferrozine

In the article describing ferrozine, Stookey (42) lists the characteristics of the reagent. These were confirmed in the initial investigations with the reagent. Ferrozine forms a magenta 1:3 iron (II) - ferrozine complex. This complex exhibits a single absorption peak in the visible spectrum with an absorptivity of 2.8×10^4 liters/mole-cm. The complex will form readily in the pH range of 4 - 9 and once formed remains intact in quite acidic solutions. Stookey (42) reports that the complex does not dissociate in 1 F perchloric acid. Further investigations showed that there was no significant change in absorbance in 0.1 F hydrochloric acid.

The number of species which react with ferrozine under the conditions used for the iron determination is quite limited. The ions of the following metals were checked: cobalt (II), nickel (II), zinc (II),

vanadium (V), copper (I), copper (II), molybdenum (VI), chromium (III), indium, gallium, lead (II), calcium, aluminum, manganese (II) and titanium (IV). Only cobalt, copper (I), nickel, and titanium showed an indication of reaction by causing a visible color. On the basis of this information it appeared that ferrozine might offer promise as a titrant in the photometric titration of iron. When the initial titrations were attempted, there was very little color produced as the titrant was added. The situation was not improved by varying the reaction conditions. It was observed that when an increment of ferrozine titrant was added to an iron (II) solution, there was, indeed, an initial small increase in absorbance. The absorbance continued to increase with time and did not reach a stable value even after 35 minutes. This behavior of the system obviously would not allow the use of ferrozine as a titrant for iron. It was hypothesized that in the situation where iron was in excess, the expected 1:3 iron - ferrozine complex did not form but one of different composition formed. The observed sluggishness of the reaction in the presence of excess iron and the almost instantaneous reaction when ferrozine was in excess might lend support to such an explanation; however, there was no confirming absorptiometric evidence. If other complexes are formed they do not cause a shift of the absorbance maximum in the visible spectra region.

The lack of success of the reagent as a titrant did not cause work with it to be abandoned, but led to further investigations in two other areas. The first of these was an attempt to improve upon the already high selectivity and sensitivity of ferrozine by application of an extraction technique. While this study was not extensive and is

basically unrelated to the main thrust of this investigation, it does offer some points of interest and will be summarized in the Appendix 2.

The second area of investigation was the application of ferrozine to the determination of iron in biological materials, specifically blood serum.

C. Importance of Serum Iron Assay

Iron is made available for transport by the serum in two ways. The first is from the breakdown of "old" red blood cells. This process occurs in the liver and is the body's mechanism of hoarding iron which is recirculated to be used in various body functions, primarily in building new red blood cells. The iron lost by the body is replaced by absorption of iron from foods. This iron in the form of iron (II) is absorbed through the mucosal cell wall of the intestine and carried by the serum to the storage points of iron in the body which are primarily in the bone marrow, liver and spleen. All iron transported by the serum is in the form of an iron (III) complex of the protein siderophilin, commonly called transferrin. The iron being transported in the form of the iron (III) transferrin complex is the species measured in serum iron determinations. This determination provides a dynamic measurement of the metabolic state of iron in the body. Other indicators of iron levels such as hemoglobin levels do not show appreciable fluctuation until there have been drastic changes in the iron reserves of the body.

In order to obtain the most complete diagnostic information it is necessary to measure not only the concentration of serum iron but also to measure the total binding capacity of the transferrin. The two pieces

of information used together provide knowledge of how much iron is being transported and how much iron the serum is capable of transporting.

The measurements of serum iron and iron binding capacity are useful aids in the diagnosis of anemia caused by hemorrhage, defective blood formation or nutrition; (ii) iron storage diseases; (iii) iron toxicity, (iv) hemachromatosis, (v) liver cell damage and others (43).

From the above list of applications for serum iron and total iron binding determinations it is quite apparent that this is an important and often used clinical assay. As such it is necessary to be able to perform large numbers of these determinations with good precision and accuracy. In addition, since the samples often come from small children, the sensitivity of the determination should be as great as possible in order to keep sample requirements at a minimum. With these thoughts in mind it was decided to put the main emphasis on the application of ferrozine to automated techniques for the determinations of serum iron and iron binding capacity.

Ferrozine offers a number of distinct advantages as a reagent for the serum iron determinations particularly with automated techniques. The most prominent of these are:

1. Sensitivity. The ferrozine-iron (II) complex shows a molar absorptivity of 2.8×10^4 liters/mole-cm which compares very favorably with 2.4×10^4 for TPTZ (2,4,6-tripridyl-s-triazine) and 2.2×10^4 for bathophenanthroline disulfonic acid, two of the most commonly used reagents for serum iron.

2. Solubility. Ferrozine and its iron (II) complex are quite soluble in water. This property simplifies handling, especially in auto-

mated techniques where certain organic solvents cause problems with the pump and transfer tubing.

3. Cost. Other sensitive reagents for the determination of iron are almost prohibitively expensive for use in flow-type rapid analysis. Ferrozine, however, costs less than $1 per gram when bought in quantities of 25 grams and more.

D. Other Procedures

There is a large number of approaches available for the determination of serum iron. These are almost universally similar, most using the AutoAnalyzer system with bathophenanthroline or TPTZ (44-48). This is not the place to compile a complete list of available procedures; those presented merely serve as examples of the approaches used. All of the procedures show a need for improved sensitivity, since either unsatisfactorily large samples must be used or if a micro-procedure is applied then high demands are placed on electronic scale expansions to obtain usable output signals.

During the course of these investigations workers reported on the application of ferrozine to serum iron determinations (49-52) including one to an automated procedure. However, this latter procedure fails to provide the best combination of conditions to prevent sample to sample carryover and does not make full use of the sensitivity inherent to ferrozine.

CHAPTER II

EXPERIMENTAL

A. AutoAnalyzer Procedure

1. Reagents and Solutions

All water used for the preparation of solutions and for washing glassware should be as iron free as possible. This can be achieved by the exclusive use of doubly distilled or deionized distilled water. In addition all water should be checked by taking a sample, adding ascorbic acid and ferrozine solutions, and measuring against ferrozine solution in a spectrophotometer at 562 nm with a 1 cm pathlength. If a reading of 0.05 absorbance units or greater is observed the water should not be used.

All reagents used should be reagent grade or better.

a. Ascorbic Acid, 2% (w/v). Place two grams of l-ascorbic acid in a 100-ml volumetric flask, and bring the volume to mark with 0.1 hydrochloric acid. Prepare this solution daily.

b. Hydrochloric Acid, 1 F. To 200 ml water in a 1-liter volumetric flask add 83 ml of concentrated hydrochloric acid and dilute to mark.

c. Hydrochloric Acid, 0.1 F. Quantitatively dilute the 1 F stock 1 to 10.

d. Acetate Buffer, 1 F. Mix 60 ml glacial acetic acid and 40 grams of 50% sodium hydroxide solution with 800 ml water. If necessary

adjust the pH to between 4.5 and 4.8 and bring the volume to one liter.

e. Ferrozine, 0.4%. Dissolve 2 grams of ferrozine in 500 ml of acetate buffer. Experience has shown that the purity of ferrozine varies between reagent lots with more recent preparations being of higher purity. Therefore the concentration of this reagent may need to be modified for best results depending upon the particular lot obtained.

f. Iron stock standard solution 1000 µg/l. Place 1.000 g iron wire in a 1-liter volumetric flask, add 10-12 ml concentrated hydrochloric acid, and dissolve with slight warming. After dissolution is complete, cool to room temperature and fill to mark. This solution is stable indefinitely when stored, well-stoppered, in a pyrex container.

g. Iron working stock solution, 100 mg/l. Dilute 10.0 ml of the 1000 mg/l stock solution to 100 ml. Prepare weekly.

h. Working standards. To obtain standards of 50, 100, 200 and 300 µg/100 ml place 1, 2, 4 and 6 ml of the 100 mg/l stock solution in 200-ml volumetric flasks add 3 to 5 drops of concentrated HCl and dilute to mark. Prepare daily.

2. Instrumentation

Technicon AutoAnalyzer

The AutoAnalyzer produced by Technicon Instruments Incorporated is one of the most widely used of all automated analytical devices. It operates on an hydraulic principle in which a peristaltic pump picks up sample, and reagents which are mixed at selected times and then pumped through a cuvet where the absorbance of the solution is measured. Air bubbles are introduced into the flowing streams to separate the samples, facilitate mixing and to scrub the tubing walls to cut down on sample

to sample carryover. This system has some unique features, primary among which is the fact that by matching the flow of two liquid streams on either side of a semipermeable membrane it is possible to dialyze a sample. This feature allows small molecules such as iron to be separated from a serum protein matrix without a manual protein precipitation step. The length of the path through a single stage dialyzer, as used in these experiments, is approximately 25 meters. Dialysis efficiency can be increased by double dialysis or placing two dialyzer stages in series. However, it should be noted that with increased dialysis efficiency the greater length of travel by the solutions gives more opportunity for intersample mixing, and as a result more carryover.

3. Analyses

The procedure as developed in this laboratory gives 100% of steady state absorbance with a 300 μg/100 ml iron standard and with serum standards at the 200 μg/100 ml level. Replicate side by side analyses of high and low iron concentrations using sera and standards showed no detectable carryover at a sampling rate of 40 per hour. The sensitivity of the procedure is indicated by the 300 μg/100 ml standard which gives an absorbance reading of approximately 0.35. This sensitivity can be approximately doubled by making minor modifications in the manifold, changing from single to double dialysis, and decreasing the rate of analysis to 20 samples per hour.

A flow diagram for the AutoAnalyzer procedure is shown in Figure 13. Samples are processed at the rate of 40 per hour with a standard 2:1 cam. With a 0.045 I.D. sample line the volume of sample used is 0.8 ml. The sample is added to the ascorbic acid - hydrochloric acid

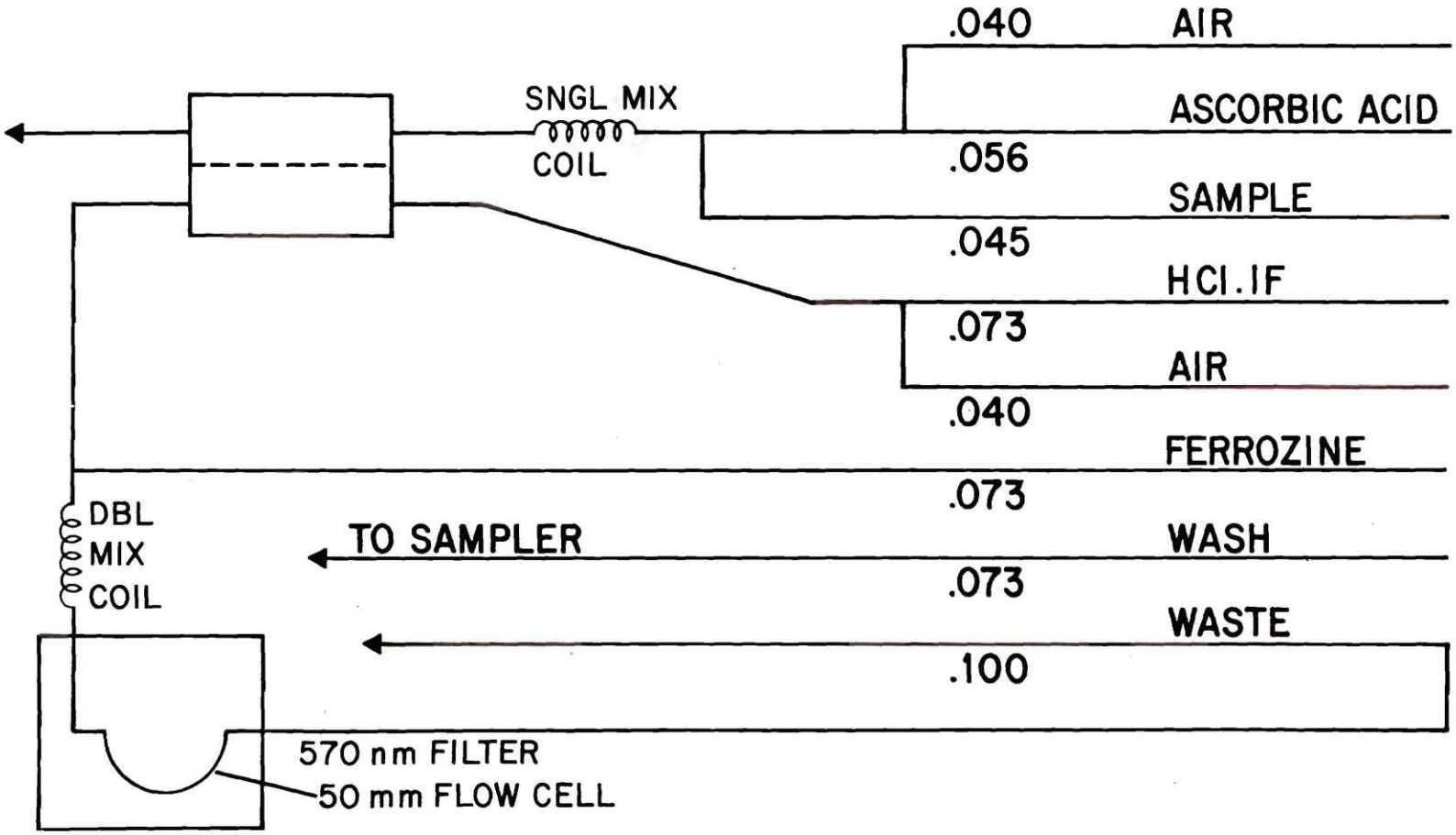


Figure 13. Flow Diagram for the Auto Analyzer Procedure for Serum Iron.

stream and mixed in a single mixing coil. This stream is then dialyzed against 0.1 F HCl in a single dialyzer. The dialysate is mixed with the ferrozine reagent in a double mixing coil. The resulting solution passes into the colorimeter where the absorbance is measured at 570 nm in a 50 mm flow cell.

Of the filters available the one closest to the 562 nm maximum of the ferrozine-iron (II) complex had a peak transmittance at 570 nm. The deviation from the optimum wavelength caused a decrease of approximately 5% in sensitivity. A 50-mm flow cell was used in place of the standard 15-mm cell to obtain adequate sensitivity without electronic scale expansion for micro samples. It was found that in routine assays the larger path length allowed a combination of greater sample dilutions and single dialysis, which in turn gave good washout at a rate of 40 samples per hour.

Studies were made on sample carryover approac of standards and samples to steady state absorbance values, adherence to Beers law by standard and samples over the analytical range chosen, and interferences, especially that of copper.

Repeated carryover checks were made in the 200 to 100 μg/100 ml range and no carryover was detected. Over the concentration span of 50 tc 300 μg/100 ml standard and sample absorbance values were the same as those observed under steady state ccnditions. When observed absorbance values of standards were plotted against concentration, a straight line resulted. A similar plot based on a series of dilutions of a serum sample also yielded a straight line.

The only metal ion commonly found in serum which can interfere

with the iron determination is copper. This interference is reported by Yee and Zin (47) and Carter (48). In the present study samples were spiked with up to five times the normal serum copper level and analyzed under routine conditions. The results for these samples and the unspiked samples agreed within the experimental errors. Therefore it was concluded that copper at normal levels would not cause interference.

The effect of hemolysis was checked by adding lysed, red cell concentrate to the serum. No interference was encountered, with hemoglobin levels in excess of 1%. When the serum was mixed with equal volumes of the cell concentrate corresponding to lysed whole blood, an increase in absorbance was observed.

To check the precision of the technique, two sera were analyzed repeatedly over a period of 5 days. Duplicate runs were made each day. Table 8 shows the results.

B. Discrete Sample Analyzer (DSA) 560 Procedure

The high sensitivity and the almost instantaneous formation of the ferrozine iron (II) complex led to the investigation of the determination of iron directly in serum without prior removal of protein. To satisfy needs for handling large numbers of samples, the DSA was chosen as the processor. This instrument combines the features of a high rate of analysis (120 per hour), low sample and reagent requirements, and a direct printout in concentration units.

1. Reagents and Solutions

The reagents and solutions used with the DSA procedure are the same as those used in the AutoAnalyzer procedure except that the ascor-

Table 8. Summary of Results Obtained on the AutoAnalyzer on Two Pooled Sera Tested Over a Period of 5 Days

	Normal	Elevated
Mean	127.7 μg/100 ml	238.6 μg/100 ml
Total determinations	121	122
SD[a]	±3.3 μg/100 ml	±3.5 μg/100 ml
RSD[b] within run	±2.6%	±1.5%
SD[c]	±4.9 μg/100 ml	±5.7 μg/100 ml
RSD[b]	±3.8%	±2.4%

a. Standard deviation of replicate determinations in the same run.

b. Relative standard deviation, (SD ÷ mean) x 100.

c. Total standard deviation of a single determination.

bic and hydrochloric acid solution are 4% and 0.2 F, respectively. These changes are necessary because the DSA pump delivers the sample or reagent along with a water wash. In this procedure, the wash volume for all reagent solutions was equal to that of the reagent solutions themselves.

2. Instrumentation

Discrete Sample Analyzer 560

The DSA 560 is produced by Beckman Instrument Company, Fullerton, California. This instrument is but one of a large number that approach automation by mechanically reproducing the actions of an analyst. In this instrument a special reaction container called a Q-cup is transported by a chain drive through a number of positions. The cup is halted at each position for a defined period and then procedes to the next position. At the first position a sample aliquot is picked up and placed in one of the five cavities in the Q-cup by a pump. The sample delivery is followed by a water wash of the probe to prevent sample carryover. At each following position it is possible to add reagents up to the final position where the sample and blank are drawn into a colorimeter and held while a differential absorbance reading is made. The absorbance is then electronically converted to concentration and the result printed on a teletype.

Because of its transport technique it is possible to provide reagent addition at exact times and to incubate between reagent additions. In the case of the reaction studied here, identical aliquots were taken; one was treated with color reagent and the other served as the serum blank.

3. Analyses

The scheme for reagent additions on the DSA is shown in Figure 14. 100 µl of sample and 150 µl of water are added to positions B and C of the Q-cup. Then 300 µl of the ascorbic acid solution is added to each of the two positions. The mixtures are allowed to react for 5 minutes at ambient temperature; then 300 µl of ferrozine solution is added to position B and 300 µl of acetate buffer to position C. After another 5 minutes, positions B and C are sampled, the differential absorbance is read, and results are printed out in concentrations units.

As in the AutoAnalyzer procedure, an optimal 560 nm filter was not available; hence the 550 nm filter, which is standard in the instrument, was used.

Although the 560 nm filter would have given a better signal-to-noise ratio, the conditions as described are adequate for routine analyses. Standards in the concentration range of 50-300 µg/100 ml iron (II) showed a linear relationship between concentration and instrument readout. Linearity was also checked by diluting a 220 µg/100 ml serum sample with saline solutions to the following fractions of the original concentrations: 1, 0.75, 0.50 and 0.25. A plot of concentration versus instrument readout again was linear.

Interference studies performed for the DSA procedure were similar to those carried out for the AutoAnalyzer. By analyzing assayed serum samples and comparing results obtained by Atomic Absorption and by the DSA on identical samples, it was determined that normal copper levels cause no detectable interference. Aqueous solutions and spiked serum pools were prepared so that they contained 500 µg/100 ml copper. An

Reagent	Sample		4% Asc. Acid in 0.2 FHCl		4% Asc. Acid in 0.2 FHCl		4% Ferrozine in Acetate Buffer		Acetate Buffer	
Pump Number	I		II		III		IV		V	
Delivered Volume µl	100	150	300	300	300	300	300	300	300	300

Total volume 1:45 ml

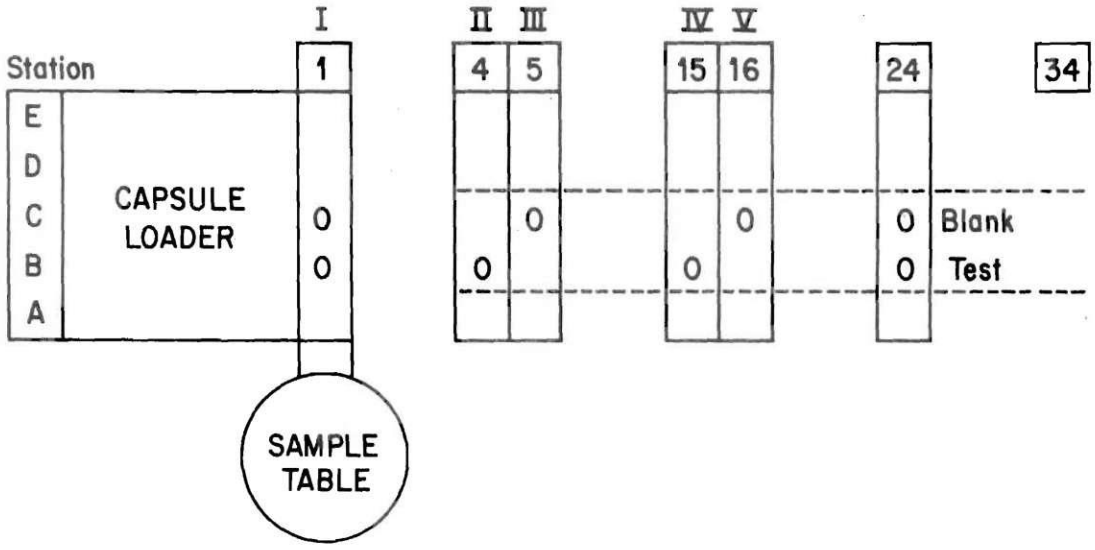


Figure 14. Scheme for Reagent Addition for the DSA Procedure for Serum Iron.

elevation in apparent iron level was observed for these solutions. This elevation was due to the formation of the copper (I) ferrozine complex.

It was found that copper interference in solutions containing only iron standard and copper could be eliminated in either of two ways.

1. After the addition of the ferrozine reagent a large excess of thiourea is added. A thiourea copper (I) complex is formed and the copper is masked against ferrozine.

2. After the addition of the ferrozine reagent, the pH of the solution is adjusted to between 0.5 and 1. This pH lowering destroys the copper ferrozine complex but does not affect the iron complex in aqueous solutions.

When these techniques are applied to serum, both eliminate the absorbance due to copper, but the second is less satisfactory because it lowers apparent iron values by about 10%. Other possible interfering agents such as hemoglobin and salicylate had no apparent effect. However, EDTA at a concentration of 150 mg/100 ml causes approximately a 25% decrease in the iron value.

An indication of the precision of the DSA procedure is given by the results of tests performed routinely over a 6-month period on two control pools. These data are shown in Table 9. A certified commercial serum preparation "Metrix" was analyzed and the results shown in Table 10 were obtained. It is felt that, if adequate standards and controls are used, single sample determinations by the DSA procedure are satisfactory for most applications.

Table 9. Summary of Results Obtained With the Beckman 560 DSA on Two Control Pools Run Over a 6-Month Period

	Normal	Elevated
Mean	135.4 μg/100 ml	222.6 μg/100 ml
Number of determinations	270	273
SD[a]	±2.1 μg/100 ml	±1.9 μg/100 ml
RSD[b]	±1.5%	±0.9%
SD[c]	±4.4 μg/100 ml	±4.7 μg/100 ml
RSD[b]	±3.2%	±2.1%

a. Standard deviation of replicate determinations in the same run.

b. Relative standard deviation, (SD ÷ mean) x 100.

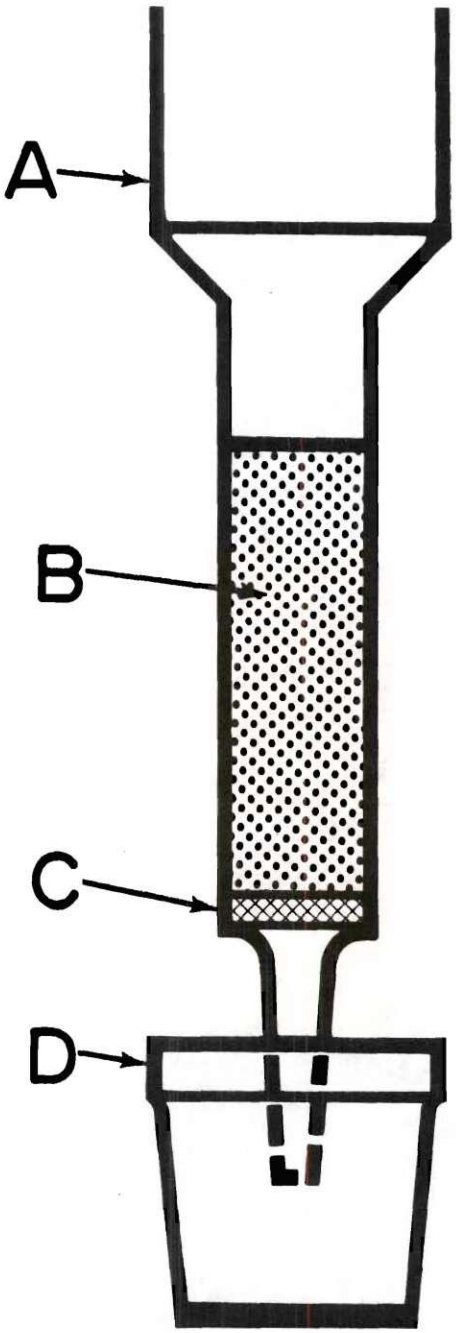
c. Total standard deviation of a single determination.

Table 10. Beckman 560 DSA Results and the Label Values for a Commercial Serum

	DSA		Label Value	
	Normal	Elevated	Normal	Elevated
Mean	102.0 μg/100 ml	295.1 μg/100 ml	101	293
Number of determinations	13	9	-	-
SD	±1.2 μg/100 ml	±2.7 μg/100 ml	-	-
RSD	±1.2%	±.9%	-	-

C. Total Iron-Binding Capacity

The general procedure for determining total iron-binding capacity (TIBC) is to saturate the transferrin present with ferric iron, remove the excess ferric iron, and perform the normal iron assay. A number of approaches has been taken to the problem of removing excess ferric iron, including absorption on ion exchange resin (53) or resin treated filter paper (44). In this study, light magnesium carbonate was used as an adsorber. About 0.2 gm $MgCO_3$ was placed in a disposable ion exchange column (Whale Scientific Inc.) into which had been inserted a disk of Whatman #3 filter paper (Figure 15). One ml of the 200 μg/100 ml standard was added to 0.5 ml of serum; the solution was mixed allowed to stand for at least 5 minutes, and then poured onto the column. The column was inserted into a capped AutoAnalyzer cup and centrifuged at low speed (750-1000 rpm) for 5 minutes. The column and cap were discarded, and the sample was analyzed for iron by either of the described procedures. For the DSA procedure the volumes of iron (III) standard and serum are halved. The result obtained is multiplied by 3 to give TIBC. This technique saves sample (a serum iron and TIBC can be performed on less than 1 ml of serum on the DSA), eliminates long high-speed spinning, avoids the problem caused by decanting from $MgCO_3$, and speeds up the overall procedure for TIBC assays. In comparisons with conventional techniques using $MgCO_3$ no differences were observed. The precision obtained with a control pool which was repeatedly analyzed over a period of 6-months is given in Table 11. The lower precision for the TIBC results is, of course, caused by the additional sample handling and dilution steps necessary in this procedure.



A. Disposable Ion Exchange Column
B. $MgCO_3$—approximately 0.2 g.
C. Filter paper disk
D. Capped AutoAnalyzer Cup.

Figure 15. Disposable Ion Exchange Column Used for Total Iron Binding Determination.

Table 11. Summary of Total Iron Binding Capacity Test Results on a Control Pool Using the Discrete Sample Analyzer

The data were taken over a period of 6 months.

Mean	329.2 μg/100 ml
Determinations	322
SD[a]	±12.3 μg/100 ml
RSD[b]	±3.7%
SD[c]	±16.2 μg/100 ml
RSD[b]	±4.9%

a. Standard deviation of replicate determinations in the same run.

b. Relative standard deviation, SD mean.

c. Total standard deviation of a single determination.

APPENDIX B

It was decided to apply the already selective reagent ferrozine as a color reagent to iron extraction procedures as described by Doll and Specker (54) and Gagliardi and Woss (55). In these procedures iron is extracted from 6 N hydrochloric acid solution into methylisobutylketone (MIBK). This procedure allows the complete separation of iron from a given matrix along with varying amounts of other metal ions. These range from almost 100% for gallium and indium to 1 to 4% for cobalt, zinc, and copper. The combination of the extraction procedure with the selectivity of ferrozine provides an almost specific photometric determination of iron along with the possibility of concentrating the sample in the extraction step.

Since ferrozine reacts only with iron in the ferrous state a double extraction was applied.

The procedure applied is as follows:

1. Make the iron solution approximately 6 F with HCl. This can be done simply by adding an equal volume of concentrated HCl to the iron solution in a separatory funnel.

2. Extract with two, 2-ml portions of MIBK and combine the organic phases in a separatory funnel.

3. Add a spatula tip (0.1 - 0.2 grams) of ascorbic acid and 5 ml of 1 F acetate buffer pH 4.7 to the organic phase, shake, and collect the aqueous phase in a 50 ml volumetric flask.

4. Repeat step 3 and combine the second aqueous phase with the

first.

5. Add 2 ml of 0.01 F ferrozine and dilute to mark with water.

6. Measure the absorbance at 562 nm and determine the iron concentration from a previously prepared standard curve.

The standard curve obtained by carrying iron standard solutions through the extraction is linear and superimposable on a curve generated without extraction.

The degree of coextraction of cobalt and copper, two cations which are known to form ferrozine complexes, was checked. The results obtained showed approximately 1% of the original cobalt and 3% of the copper in the final aqueous phase. These results correspond well with the values of 1% and 4% reported by Doll and Specker (54). Cobalt proved resistant to masking efforts but copper (I) yielded readily to thio urea masking as described earlier in this section. Using this technique a 2,000 times molar excess of copper can be masked. Since such a small amount of cobalt coextracts with the iron if the extraction is repeated cobalt can be virtually completely removed.

Gallium and indium were also carried through the extraction and although they coextract with iron, neither react with ferrozine under the conditions applied.

REFERENCES

42. L. L. Stookey, Anal. Chem., 42 (7), 779, (1970).

43. A Brief Treatise of the Clinical Usefulness of Serum Iron, American Monitor, 1.

44. T. J. Giovanniello, G. DiBenedetto, D. W. Palmer, and T. Peters, Jr., J. Lab. and Clin. Med., 71 (5), 874, (1968).

45. B. Klein, N. Kleinman, and R. L. Searcy, Clin. Chem., 16 (6), 495, (1970).

46. P. J. Garry and G. M. Owen, Technicon Symposia, Automation in Analytical Chemistry, 1, 177, (1967).

47. D. D. Clarke, and R. Nicklas, Technicon Symposia, Automation in Analytical Chemistry, 1, 181, (1967).

48. H. S. Friedman, and C. S. Cheek, Clin. Chim. Acta, 31, 315, (1971).

49. H. V. Yee, and A. Zin, Clin. Chem., 17, 950, (1971).

50. P. Carter, Anal. Biochem., 40, 450, (1971).

51. J. P. Persijn, W. Van Der Slik, and A. Riethrost, Clin. Chim. Acta, 35, 91, (1971).

52. D. Plaut, and E. Miller, Clin. Chem., 17, 661, (1971).

53. J. D. Cook, J. Lab. Clin. Med., 76, 497, (1970).

54. W. Doll and H. Specker, Z. Anal. Chem., 161, 354, (1958).

55. E. Gagliardi, and H. P. Woss, Z. Anal. Chem., 248, 302, (1969).

VITA

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In September 1955 he entered Georgia State College, Atlanta, Georgia. He transferred to Georgia Institute of Technology in September 1956. In June 1959 he joined the Air Force Reserve and returned to Georgia Institute of Technology in September 1960. He was graduated from that school with a B.S. in Chemistry in June 1962.

In March 1962 he was employed by the Center for Disease Control and has remained with that organization to the present time.

In June 1962 he was married to Yvonne I. Burke of Decatur, Georgia. They are the parents of a son Leslie Howell, born in August 1966, and a daughter Victoria Anne, born in June 1970.

In 1967 he was selected to be supported in graduate study by the Department of Health, Education and Welfare through the Center for Disease Control. He began graduate work in the School of Chemistry, Georgia Institute of Technology in September 1967 and continued through September 1970 when he returned to full-time duties with the Center for Disease Control.